STUDY OF GDNF-FAMILY RECEPTOR ALPHA 2 AND INHIBITORY ACTIVITY OF GDNF-FAMILY RECEPTOR ALPHA 2B (GFRα2B) ISOFORM

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Abstract

The glial cell-line derived neurotrophic factor (GDNF) and neurturin (NTN) belong to a structurally related family of neurotrophic factors. GDNF and NTN exert their effects through a multi-component receptor system consisting of the GDNF family receptor alpha (GFRα) and the co-receptor RET and/or NCAM. GDNF preferentially binds to GFRα1, while GFRα2 is the cognate receptor for NTN.

This study focused on the biochemical and morphological effects of ligand-activated GFRα1 and GFRα2 isoforms. In the initial part of the study, GDNF and NTN were found to activate distinct miRNA precursors in cells endogenously expressing RET, NCAM and GFRα2 but not GFRα1, indicative of specificity in ligand-receptor cross-talk.

There are at least three alternatively spliced isoforms of GFRα2 in the nervous system: GFRα2a, GFRα2b, and GFRα2c. Quantitation using highly specific and sensitive quantitative real-time PCR revealed comparable expression levels of these isoforms in various regions of the human brain, lending evidence to the idea that the isoforms may have physiological roles in the nervous system. These isoforms showed ligand-selectivity in MAPK (ERK1/2) and Akt signaling, and regulated different early response genes. When stimulated with GDNF or NTN, both GFRα2a and GFRα2c, but not GFRα2b, promoted neurite outgrowth in transfected Neuro2A cells. In co-expression studies, GFRα2b was found to inhibit ligand-induced neurite outgrowths mediated by GFRα2a, GFRα2c, and GFRα1a, another member of the GDNF family receptor. Furthermore, activation of GFRα2b also inhibited neurite outgrowths induced by retinoic acid and the inhibitory activities were RhoA dependent. On the other

hand, the ligand-induced neurite outgrowths through GFRα2a and GFRα2c isoforms showed distinct signaling mechanisms.

Differential biochemical and neuritogenic activities also exist with the GFRα1 receptor isoforms, GFRα1a and GFRα1b. When co-expressed, GFRα1b antagonized neurite outgrowth mediated by GFRα1a, in a RhoA-ROCK dependent manner.

The results from this study suggest a novel paradigm for the regulation of growth factor signaling and neurite outgrowth via an inhibitory splice variant of the receptor. Thus, depending on the expressions of specific GFRα2 and GFRα1 receptor spliced isoforms, GDNF and NTN may promote or inhibit neurite outgrowth through the same multi-component receptor complex. The emerging view is that the combinatorial interactions of the spliced isoforms of GFRα1, GFRα2, RET and NCAM may contribute to the complexity of multi-component signaling system and produce a myriad of observed biological responses.

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Abbreviations

ART	Artemin
CRMP	Collapsin response mediator proteins
ERK1/2	extra-cellular signal regulated kinase 1/2
FBS	fetal bovine serum
GAD	glutamate decarboxylase
GDNF	glial cell line-derived neurotrphic factor
GFL	GDNF family ligands
GFRα	GDNF family of receptors alpha
GPI	glycosyl-phosphotidylinositol
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MCS	multiple-cloning site
mESC	mouse embryonic stem cells
miRNA	microRNA
NCAM	neural cells adhesion molecules
NTN	Neurturin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PSP	Persephin
RA	retinoic acid
RET	rearranged during transformation
RhoA	Ras homologous member A
ROCK	Rho-associated kinase
RTKs	receptor tyrosine kinases
TGF-β	transforming growth factor beta (TGF-β)

Chapter 1 Introduction

1.1 Background

The glial cell-lined derived neurotrophic factor (GDNF) and neurturin (NTN) belong to a structurally related family of neurotrophic factors. GDNF and NTN exert their effects through a multi-component receptor system. GDNF and NTN bind to specific GDNF family receptors (GFRα), which are linked to the plasma membrane by a glycosyl-phosphotidylinositol (GPI) anchor. These receptors then transduce intracellular signals by activating the co-receptor, RET (a transmembrane tryrosine kinase), and/or NCAM. GFRα1 and GFRα2 are the preferred receptors for GDNF and NTN, respectively. Both ligands have potent trophic effects in many neuronal systems, including the midbrain dopaminergic neurons, making it a strong therapeutic candidate for several neurodegenerative diseases. Clinical trials I/II using GDNF and NTN transgene are currently being explored as therapeutics for Parkinson's disease.

1.2 Motivations

Despite the many efforts to unravel the biological functions of GDNF, the mechanisms underlying receptor-ligand interactions and signalings remain unclear. Our laboratory and several others have previously identified alternatively spliced isoforms of GFRα1 and GFRα2. The biological significance of these alternative spliced variants remains uncertain. Hence, it is the intention of this work to gain a better understanding of the biochemical properties, cellular functions and biological activities of the alternatively spliced GFRα2 receptor isoforms.

1.3 Objectives

This piece of work focused primarily on the study of the interactions of GDNF and NTN with the alternatively spliced GFRα2 isoforms. GFRα2 receptor is spliced to produce three isoforms, namely GFRα2a (contains all 9 exons), GFRα2b (lacking exon 2), and GFRα2c (lacking exon 2 and 3). In order to gain a better understanding of the biological significance, the expression levels of GFRα2 isoforms in different regions of the human brain were determined and their biochemical activities and phenotypical functions in inducing morphological changes, were characterized *in vitro*. The study was then extended to another structurally related family of receptors, the GFRα1 isoforms.

1.4 Organization of the thesis

This thesis is organized into five sections according to the results and findings. The first study focused on ligand-receptor specificity using a human neuroblastoma cell line that endogenously expresses the GFRα2 and the co-receptors, RET and NCAM (Chapter 3). The second section deals with the biochemical and neuritogenic activities of GFRα2 receptor isoforms using transfected Neuro2A cell models (Chapter 4). The third section deals with the mechanism underlying the neurite outgrowth inhibitory activities of the GFRα2b in more detail (Chapter 5). This is then followed by the studies of GFRα1 isoforms and demonstrations of some similarities between GFRα1b and GFRα2b on regulating neurite outgrowths inhibitions (Chapter 6). The final section (Chapter 7) focuses on the signaling differences underlying neurite outgrowth mechanisms of GFRα2a and GFRα2c isoforms. The thesis concludes with some suggestions for future works (Chapter 8).

Chapter 2 Literatures review

2.1 The neurotrophic factors

Neurotrophic factors are polypeptides that are crucial for the growth, differentiation and survival of neurons in the developing nervous system, and also play roles in functional maintenance of neurons in the mature nervous system (Blesch, 2006). Nerve growth factor (NGF) was the first neurotrophic factor discovered which was first shown to be target derived. The discovery and understanding of NGF led to the formulation of the *Neurotrophic Factor Hypothesis*, which postulates that: "*...once a developing neuron has grown its process into its target, it competes with other developing neurons of the same type for a limited supply of a neurotrophic factor provided by the target*" (Yuen *et al.*, 1996). In this hypothesis, the successful competitors for neurotrophic factor survive, while the unsuccessful ones die.

The fact that some but not all isolated neurons responded to NGF, led to the speculation that there are likely to be more neurotrophic factors and their effects should be neuron specific. Thereafter, other members of the NGF family (neurotrophins) were discovered, which include brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5).

In the early 1990s, in the pursuit to discover dopaminergic neuron specific supporting factors, glial cell line-derived neurotorphic factor (GDNF) was purified from culture supernatants of the glial cell line B49 and the gene cloned (Lin *et al.*, 1993). Other GDNF family members, Neurturin (Kotzbauer *et al.*, 1996), Artemin (Baloh *et al.*, 1998) and Persephin (Milbrandt *et al.*, 1998) were subsequently identified and the genes cloned. More than one decade after the discovery of GDNF, the continuing efforts and interests are now focusing on understanding the functions and signaling mechanisms of this family of ligands (GDNF family of ligands, GFLs)

and receptors (GDNF family of receptors alpha, GFRα) in neuronal and non-neuronal systems.

2.2 GDNF family of ligands (GFLs)

Glial cell-line derived neurotrophic factor (GDNF), Neurturin (NTN), Artemin (ARTN) and Persephin (PSPN) are cysteine-knot proteins and are structurally related neurotrophic factors (Airaksinen and Saarma, 2002; Kobori *et al.*, 2004). These GFLs have been shown to support the growth, maintenance and differentiation of a wide variety of neuronal and extra-neuronal systems (Saarma and Sariola, 1999). Structurally, GFLs belong to the transforming growth factor beta (TGF-β) superfamily, sharing the seven conserved Cys residues (depicted in Figure 1.1). GFLs are biosynthesized as precursors and further processed into the mature forms of disulfide-bonded dimeric, basic and secretory proteins.

GDNF and the other GFLs act as trophic factors for many central and peripheral neuronal systems, such as the sensory, enteric, sympathetic, and parasympathetic (Airaksinen and Saarma, 2002; Airaksinen *et al.*, 1999). GDNF also has functions in some non-neuronal systems, such as in kidney development and spermatogonial differentiation (Sariola, 2001; Sariola and Saarma, 1999). Because GDNF has potent neurotrophic effects on midbrain dopaminergic neurons and other neuronal systems, it is perhaps not surprising that GDNF is considered a useful therapeutic for some neurodegenerative diseases. Indeed, GDNF has been used in clinical trials and the results are favorable in some reports (Gill *et al.*, 2003; Slevin *et al.*, 2005) but not in others (Nutt *et al.*, 2003; Peggy, 2005). It is now believed that the failure of some of these clinical trials may simply be technical variability resulting in the suboptimal bioavailability of GDNF (Salvatore *et al.*, 2006) and statistical errors (Hutchinson *et al.*, 2006). The difficulty of delivering large proteineous factors may one day be

circumvented by the use of small molecular mimetics which show biochemical properties similar to GDNF and the other GFLs (Bespalov and Saarma, 2007).

Although NTN and GDNF are structurally related, the tissue distributions of their cognate receptors do not share significant overlaps, indicative of possible distinct functional roles (Golden *et al.*, 1999; Widenfalk *et al.*, 2000). When compared to GDNF, the chronic administration of NTN produces specific neurochemical changes only in the ventrolateral striatum with no detectable adverse effects, raising the possibility that NTN may also serve as a useful therapeutic (Hoane *et al.*, 1999). A Phase I clinical trial using an *in vivo* Adeno-associated Virus Type 2 (AAV2) mediated delivery of the gene encoding NTN (CERE-120) is currently underway (http://www.clinicaltrials.gov).

With the better understanding of structure-functions of the molecules, physiological roles and signaling mechanisms of GFLs, it may enable the rational development of efficacious therapeutics for diseases related to this family of ligands and receptors.

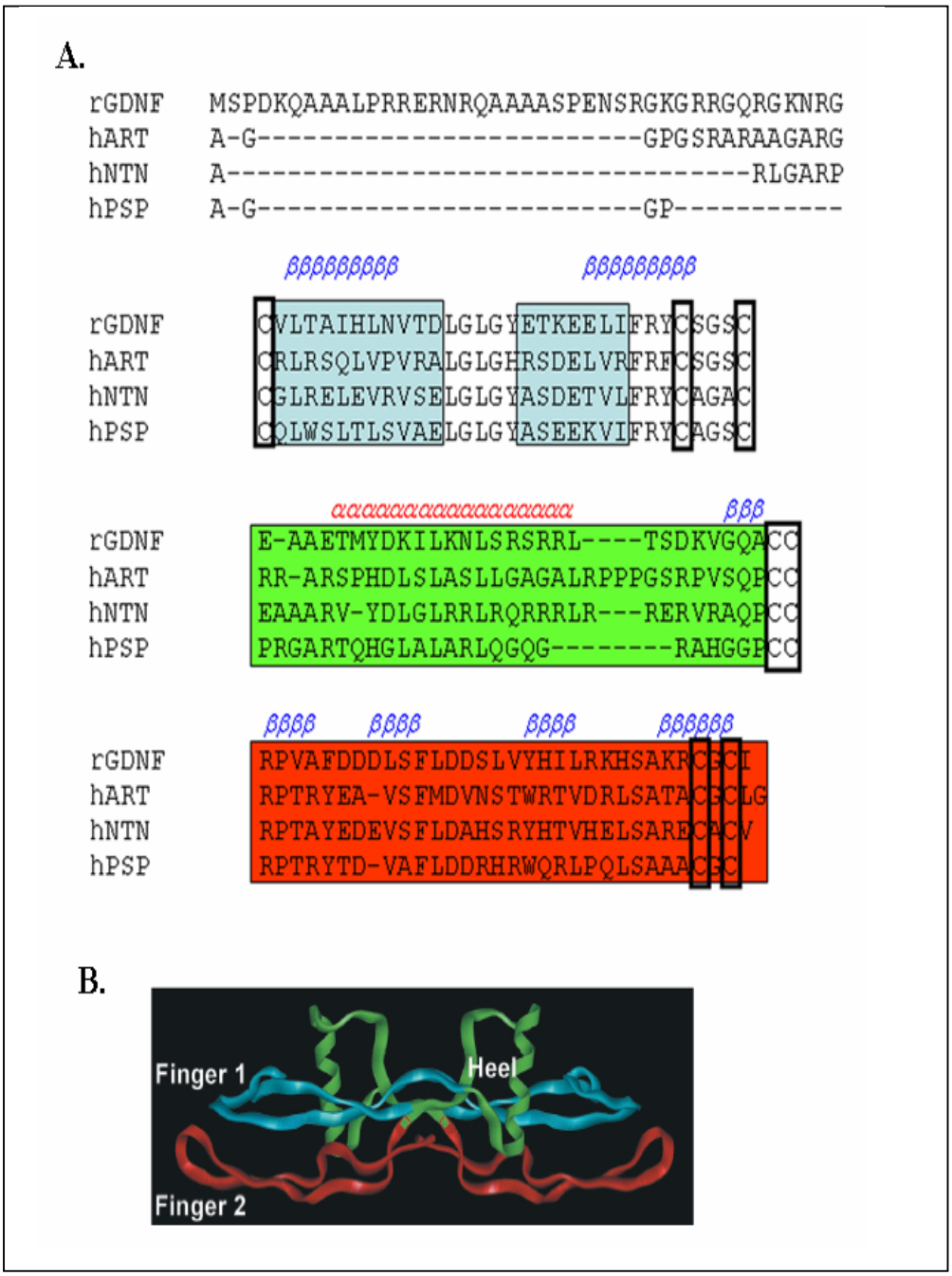

Figure. 1.1 Amino acids sequence alignment of mature GDNF family ligands (GFL). A, Amino acids sequence alignment of rat GDNF, human Artemin (hART), human Neurturin (hNTN), and human Persephin (hPSP). Boxed regions show position of the conserved cysteine residues. Secondary structure elements are indicated above the alignment ("α" for α-helix; "β" for β strand). Regions in color correspond to color scheme shown in ***B.*** **B,** Representation of backbone of the GDNF dimer. The first (blue) and second (red) fingers, and the heel (green) region of the molecule are shown. Figure B, adapted from Baloh *et al*, 2000.

2.3 GDNF family receptors

GFLs exert their effects through a multi-component receptor system consisting of the GDNF family receptor alpha (GFRα), RET (rearranged during transformation) and/or NCAM (neural cells adhesion molecules) (Airaksinen *et al.*, 1999; Paratcha *et al.*, 2003). The GFRα family consists of four members (GFRα1 to 4) that are linked to the plasma membrane via glycosyl-phosphotidylinositol (GPI) anchor. The homologs of the genes encoding these receptors are found in different chromosomes (Table 1.1). GFRα receptor family members share 30-45% amino acid identity, with similar conserved cysteine residue arrangements (Figure 1.2), suggesting that their secondary structures may also be conserved (Scott and Ibanez, 2001). A comparison of these GFRα receptors amino acids reveals internal structural homologies within the conserved cysteine rich sequences, suggesting common putative domain structures for these receptors.

Table 1.1 Chromosome locations of *Mus muculus* and *Homo sapiens* GFRα receptors genes. The genetic loci are described in the NCBI Mapviewer, build 36 for both organisms.

	Gene of receptor	Chromosome Location
Mouse	GFRα1	19 D2-D3; 29.0 cM
	GFRα2	14 D3-E1
	GFRα3	18 B1
	GFRα4	2 F1 73.9 cM
Human	GFRα1	10q26
	GFRα2	8p21.3
	GFRα3	5q31.1-q31.3
	GFRα4	20p13-p12

Figure 1.2 Amino acid sequence comparison of GFRα1, GFRα2, GFRα3, and GFRα4. The amino acid sequence of rat GFRα1, GFRα2, human GFRα3, and chicken GFRα4 are aligned and the conserved cysteines are boxed in red. Sequences predicted to correspond to α helix (blue) and *β* strand (purple) are highlighted. Predicted N-terminal signal peptide sequences and the C-terminal hydrophobic regions are underlined. Figure modified from Scott and Ibanez, 2001.

Each GFL is known to bind itself to a preferential GFRα receptor (depicted in Figure 1.3). GFRα1 is found to be the cognate receptor for GDNF (Jing *et al.*, 1996; Treanor *et al.*, 1996). NTN signals through its preferred receptor GFRα2 (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Klein *et al.*, 1997; Widenfalk *et al.*, 1997). Artemin and Persephin signals through GFRα3 and GFRα4, respectively.

Upon ligand binding to these GFRα receptors, intracellular signals are transduced through the trans-membrane receptor tyrosine kinase, RET. Recent findings suggest that NCAM may also function as the co-receptor for GFLs-GFRα signaling (Paratcha *et al.*, 2003), adding to the complexity of the signaling mechanism of GFLs and GFRα.

The key role of GDNF and its receptor GFRαl in enteric nervous system development is conserved from zebrafish to humans. The role of Neurturin, signals via GFRα2, for parasympathetic neuron development is also conserved between chicken and mice. The role of Artemin and Persephin that signals via GFRα3 and GFRα4, respectively, is currently unknown in non-mammals. Recent phylogenetic study (Airaksinen *et al.*, 2006; Hatinen *et al.*, 2006) indicates that orthologs of all four GFL are present in mammals, as well as in bony fish (teleost) (Figure 1.4A). Orthologs of all GFRα receptors are also present in all vertebrates classes, from bony fish to mammals (Figure 1.4B). However, Persephin is missing from chicken genome, while frog genome lacks ortholog of Neurturin (Hatinen *et al.*, 2006), suggesting functional redundancy in early tetrapods. The functional significance of non-mammalian GFLs and GFRα signaling remains unclear.

Recently, distantly related GFRα-like structures have been identified. Based on the conserved pattern of cysteines (and the presence of some amino acid residues), these sequences include Gas1, growth arrest specific 1 protein, (Cabrera *et al.*, 2006;

Schueler-Furman *et al.*, 2006) and GRAL (GDNF Receptor Alpha Like), a protein found in some regions of the central nervous system of unknown function (Li *et al.*, 2005). In addition, genomic sequences encoding a predicted protein in echinoderm sea urchin (*Strongylocentrotus purpuratus*) that shows clear homology to vertebrate GFRα and GRAL proteins but no known function has been identified and this hypothetical protein is called GDNF family receptor-like (GFRL) (Hatinen *et al.*, 2006). Unlike the GFRα1-4, GRAL and Gas 1 function independently of GFLs. Hence, these related proteins may have distinct ligands which are not GFLs.

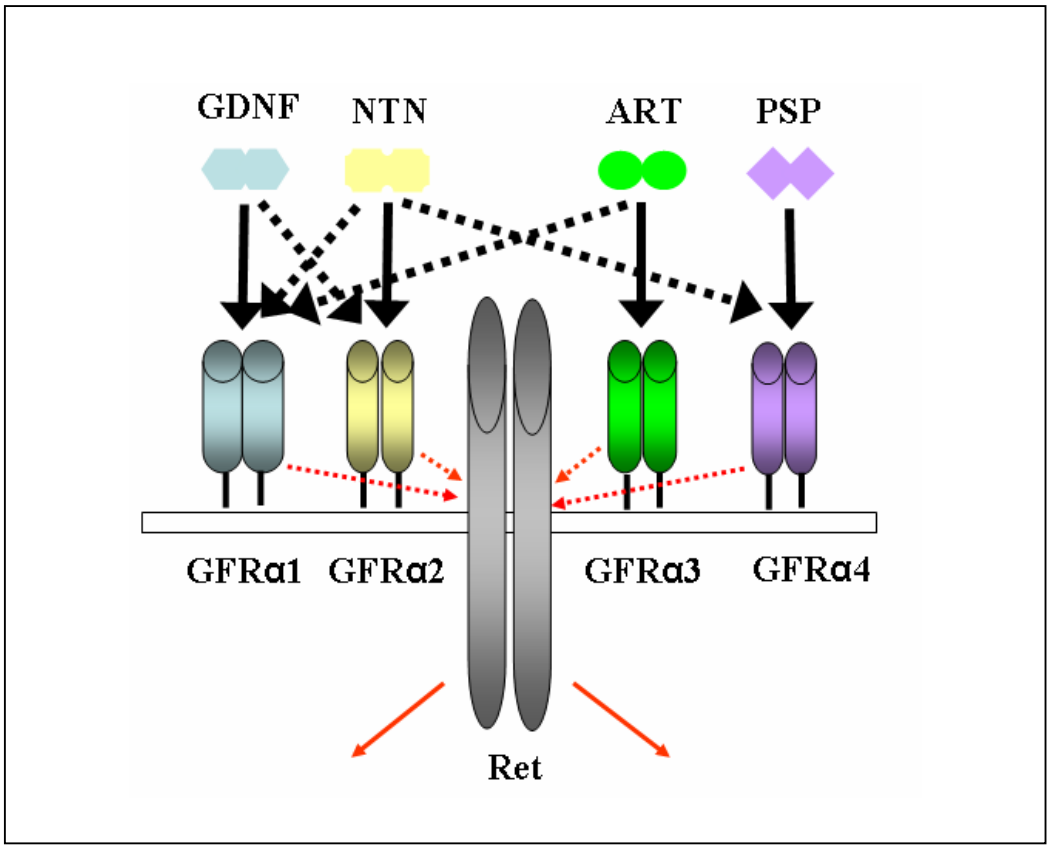

Figure 1.3. Schematic diagram of GFLs binding to GFRα receptors. GDNF, NTN (Neurturin), ART (Artemin), and PSP (Persephin) bind to preferred GFRα receptors (indicated by solid, black arrows), and activate (indicated by dashed, red arrows) transmembrane Ret tyrosine kinase receptor to transduce intracellular signaling (indicated by solid, red arrows). Promiscuous binding between GFL and non-preferred receptors are also shown (dotted, black arrows).

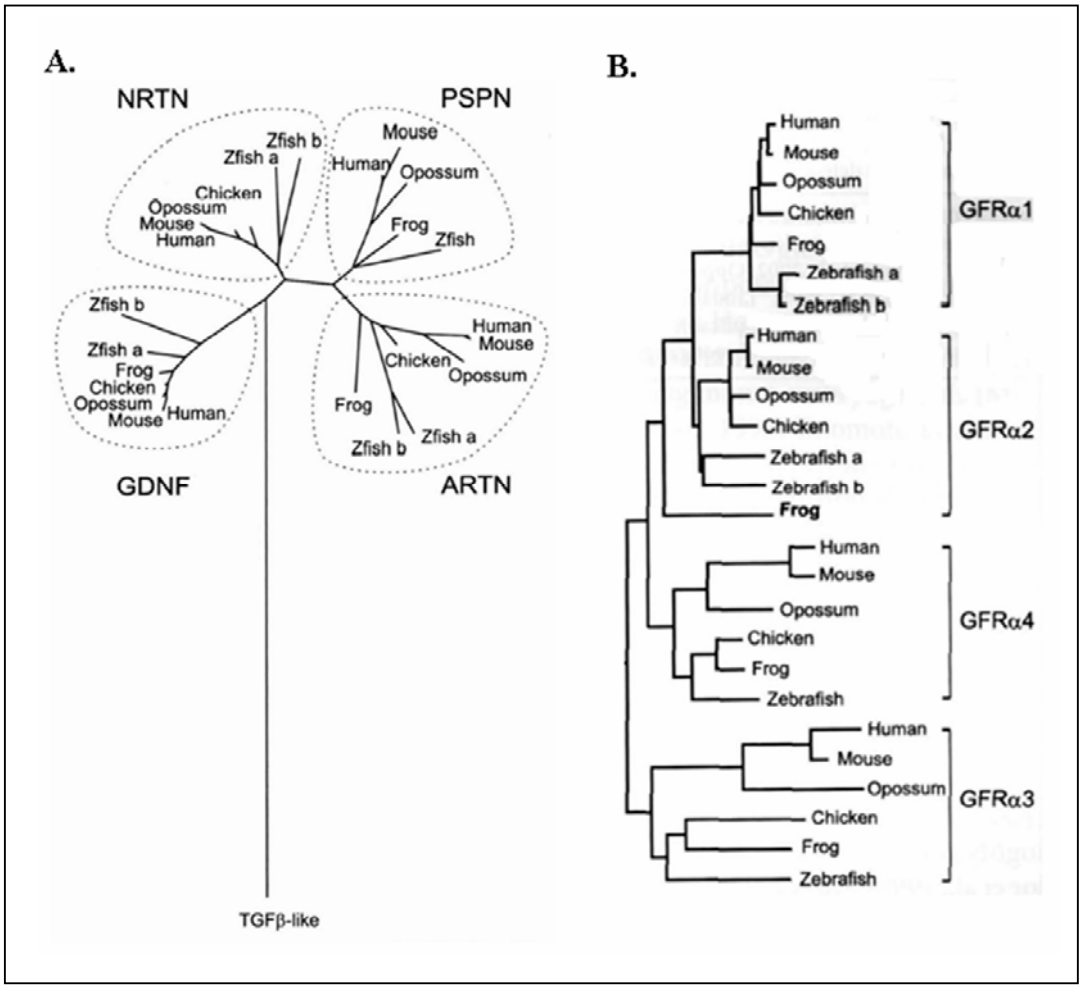

Figure 1.4. Phylogenetic analysis of GDNF Family Ligands (GFL) and GFR superfamily proteins. A, The tree was generated by comparing the mature part of the GFLs (NRTN for Neurturin, PSPN for Persephin, and ARTN for Artemin) using the maximum likelihood method. Threadworm (*Strongyloides stercoralis*) TGFP-like protein was used as the outgroup. Note the absence of PSPN in chicken and NRTN in clawed frog. **B,** Phylogenetic tree of GFR superfamily proteins in selected animal species. The tree was generated by comparing the conserved part of the proteins. The branches lengths are proportional to the expected proportion of amino acid differences among groups. Figures adapted from Airaksinen *et al*, 2006.

2.4 Alternatively spliced isoforms of GFRs and their co-receptors

Alternative splicing is prevalent in many mammalian genomes and is a means of producing functionally diverse polypeptides from a single gene (Blencowe, 2006). Recently, genome-wide microarray and large-scale computational analyses of expressed sequence tag and cDNA sequences have estimated that greater than 50% of human multi-exon genes are alternatively spliced (Modrek and Lee, 2002). Comparative genomic analyses further demonstrate that the greatest amount of conserved alternative splicing occurs in the central nervous system (Kan *et al.*, 2005). In many systems, alternative splicing events have been shown to produce isoforms with distinct activities and biochemical properties as a means for diverse biological functions (Lee and Irizarry, 2003).

Multiple alternatively spliced variants of GFRα1 (Dey *et al.*, 1998; Sanicola *et al.*, 1997; Shefelbine *et al.*, 1998), GFRα2 (Dolatshad *et al.*, 2002; Wong and Too, 1998) and GFRα4 (Lindahl *et al.*, 2001; Lindahl *et al.*, 2000; Masure *et al.*, 2000) have been reported. The alternatively spliced isoforms of GFRα1 have been shown to exhibit distinct biochemical functions (Charlet-Berguerand *et al.*, 2004; Yoong *et al.*, 2005). Similarly, alternatively spliced isoforms of the GFRα co-receptors, RET (de Graaff *et al.*, 2001; Lee *et al.*, 2002a; Lorenzo *et al.*, 1997) and NCAM (Buttner *et al.*, 2004; Povlsen *et al.*, 2003) have been reported. Ret9 and Ret51 are the two spliced isoforms of RET, both of which have been shown to possess distinct biochemical and physiological functions (de Graaff *et al.*, 2001; Lee *et al.*, 2002a; Lorenzo *et al.*, 1997). These observations are consistent with the emerging view that the combinatorial interactions of the spliced isoforms of GFRα, RET and NCAM may contribute to the multi-component signaling system in producing the myriad of observed biological responses. The existence of multiple splice isoforms of GFRα,

RET and NCAM and the possible combinatorial interactions of these spliced isoforms will invariably increase the complexity of the signaling of this multi-component system. This complexity is further increased by the existence of cross talks of different GFLs with the same GFRα isoform.

2.5 GFRα2 and GFRα1 receptor

At least three alternatively spliced isoforms of GFRα2 receptor have been identified in mammalian systems, namely GFRα2a (1393 bp), GFRα2b (1077 bp) and GFRα2c (993 bp) (Dey *et al.*, 1998; Sanicola *et al.*, 1997; Shefelbine *et al.*, 1998). GFRα2 isoforms differ only in their N-terminal, with GFRα2b lacking exon 2 (of total 9 exons), and GFRα2c lacking exons 2 and 3. All three isoforms have been detected in various human and murine tissues (Too, 2003; Wong and Too, 1998) as well as in the rat myenteric plexus (Dolatshad *et al.*, 2002).

GFRα1 has previously been shown to respond to GDNF and NTN (Pezeshki *et al.*, 2001; Wang *et al.*, 2000), with preferential pairing to the former (Baloh *et al.*, 2000; Creedon *et al.*, 1997). GFRα1 is spliced to produce two isoforms, namely the GFRα1a and GFRα1b (Dey *et al.*, 1998; Shefelbine *et al.*, 1998). These 2 isoforms are highly homologous, with a difference of only five amino acids (140DVFQQ144), and lacking in GFRα1b. GFRα1a appears to be structurally organized into 3 distinct domains (Eketjall *et al.*, 1999). The Domain 3 (residues 239-346) of GFRα1a has been crystallized and used to model Domain 2 (Leppanen *et al.*, 2004). Interestingly, the predicted Domain 2 (residues 150-238) helices (Airaksinen *et al.*, 1999) show the same positions of cysteine residues which are thought to form disulfide bridges, as observed in the helices in Domain 3. Both Domain 2 and 3 are involved in the binding

of GDNF. A similar structural organization of GFRα3a has also been proposed based on crystal structure of Artermin- GFRα3 ectodomains 2 and 3 (Wang *et al.*, 2006). It is now generally believed that the GFRαs share such structural organizations.

Based on the structural organization, Domains 1 and 2 of the GFRα are thought to be linked by an extended loop (residues 114-144). Interestingly, the smaller spliced isoforms of GFRα1 (GFRα1b) and GFRα2 (GFRα2b and GFRα2c) showed exon deletions which reside in Domain 1. The absence of the five amino acids (140DVFQQ144) in GFRα1b isoform or the deleted 5' exons in GFRα2b and GFRα2c may confer significant structural differences between the spliced isoforms and resulting in different functional consequences. It will be of great interest in the future if Domain 1 of GFRα1a and GFRα2a can be structurally determined along with the other ligand binding domains (Domain 2 and 3) for a more precise definition of the receptors as a whole.

Chapter 3 Part I: Glial cell-line derived neurotrophic factor and Neurturin regulated the expressions of distinct miRNA precursors through the activation of GFRα2.

3.1 Background and objectives

GFLs exert their effects through a multi-component receptor system consisting of the GFRα, RET and NCAM (Airaksinen *et al.*, 1999; Paratcha *et al.*, 2003). Each GFL is known to bind preferentially to one GFRα *in vitro* and the activation of the multi-component receptor system show some degree of promiscuity in their ligand specificities (Airaksinen *et al.*, 1999; Cik *et al.*, 2000; Horger *et al.*, 1998; Scott and Ibanez, 2001; Wang *et al.*, 2000). Mice lacking in GDNF, GFRα1 or RET share common phenotypes of kidney agenesis and the absence of many parasympathetic and enteric neurons (Cullen-McEwen *et al.*, 2001; Enomoto *et al.*, 1998; Enomoto *et al.*, 2001). Mice lacking NTN or GFRα2 show similar deficits in parasympathetic and enteric innervations but notable differences have been reported (Heuckeroth *et al.*, 1999; Rosenthal, 1999; Rossi *et al.*, 1999). These phenotypic differences may be due to different genetic background of the mice used or more interestingly, suggests the possibility of GDNF crosstalk through GFRα2 *in vivo*. GDNF has been used in clinical trials and the results were favorable in some (Gil *et al.*, 2002; Slevin *et al.*, 2005) but not in other reports (Nutt *et al.*, 2003; Peggy, 2005). These differences are currently being addressed and are likely to be due to technical differences (Salvatore *et al.*, 2006). Although cross talk in the development may not be significant (Airaksinen and Saarma, 2002), it may be highly relevant when exogenous GFLs are applied *in vivo*. It is not known if the cross talks by different GFLs with the same multi-component system produce the same biological responses. This chapter addressed this issue by examining the changes in microRNA expression when the same receptor multi-component was stimulated by two related GFLs, GDNF and NTN.

It is interesting to note that the recent studies of genome-wide transcription suggest that more of the genome are transcribed than currently annotated and much of this is noncoding. From full-length cDNA sequencing of human cDNA clones, greater than half of the transcripts found are noncoding (Ota *et al.*, 2004b). In the mouse, a large number of the FANTOM3 cDNAs lack any protein-encoding sequence and are annotated as noncoding RNAs, which out numbered the protein coding transcription units (Carninci *et al.*, 2005). With the increasing number of noncoding RNAs found, it is currently unknown if they are functional or, merely transcriptional noise. However, recent evidence suggests distinct roles of some of these transcripts in the nervous system (Cao *et al.*, 2006; Mehler and Mattick, 2006; Presutti *et al.*, 2006). Among several classes of noncoding RNAs, microRNA has been a focus of recent intense research.

MicroRNAs (miRNAs) are small non-coding RNAs that serve as important post-transcriptional regulators of gene expression in metazoan (Pillai *et al.*, 2006). To date, a large number of miRNAs have been identified in several organisms, including vertebrates and plants (Dugas and Bartel, 2004; Harfe, 2005). The number of miRNA genes appeared to be greater than 1% of the predicted genes in human (Aravin and Tuschl, 2005; Berezikov *et al.*, 2006; Lim *et al.*, 2003). To date, more than three thousand eight hundred mature miRNAs from different species have been listed in the database from Sanger Center (http://microrna.sanger.ac.uk/sequences/index.shtml), more than 400 are of human origin. In many respects, miRNA genes resemble protein coding genes in that they may possess introns (Rodriguez *et al.*, 2004) and are transcribed by RNA polymerase II (Lee *et al.*, 2004). In addition, the transcripts from miRNA genes are capped, spliced and polyadenylated (Cai *et al.*, 2004). Pre-miRNA sequences are predicted based on the folded structures and are derived from primary

transcript, pri-miRNA (Bartel, 2004). The mature miRNA (21-24 nucleotides) is located in the hairpin structure of pre-miRNA (Lee *et al.*, 2002b) . This maturation process is highly regulated (Thomson *et al.*, 2006; Zeng, 2006). Biogenesis and maturation of miRNA involved a few stages. In the nucleus, primary miRNA transcript (pri-miRNA) is excised by an RNaseIII type endonucleus Dosha to produce a duplex RNA that contains 5' phosphate and 3' –OH, and usually with a 2 nucleotides overhang at 3' end precursor miRNA (pre-miRNA) approximately 60-70 nucleotides long. The pre-miRNA is then exported to cytoplasm by Exportin 5 (Exp 5) and further cleaved by another RNaseIII type endonucleus, Dicer, to produce the 21-24 nucleotides miRNA duplex, with 2 nucleotides over hang at both ends (Zeng, 2006).

miRNAs have extensive regulatory roles including the involvement in development, cell proliferation, cell death, and morphogenesis (Ambros, 2003; Kasashima *et al.*, 2004; Kawasaki and Taira, 2003; Pillai, 2005; Sunkar and Zhu, 2004). A large number of these miRNAs were detected in brain, at different stages (Sempere *et al.*, 2004). The current view is that miRNAs in the nervous system may be important for cell fate decisions, neural connectivity, cell shape and adhesion, and synapse function (Presutti *et al.*, 2006).

It is currently unknown if GDNF and NTN may regulate the expression of miRNAs in various cellular processes. In this study, the human BE(2)-C cells, which expresses GFRα2 but not GFRα1, was used to examine the regulation of some miRNA precursors (pre-miRNAs and pri-miRNAs) by GDNF and NTN. Interestingly, the results showed that despite the promiscuity of ligand-receptor interaction, GDNF and NTN regulated the expression of distinct miRNA precursors through the activation of the MAPK (ERK1/2) signaling pathways.

3.2 Results

3.2.1 Neuroblastoma BE(2)-C cells express GFRα2, NCAM and RET

The quantitative real time PCR assays designed to amplify GFRα1, GFRα2, NCAM and RET were highly sensitive (detection limit of ten copies per reaction) and specific, showing only single product of the predicted size corresponding to each amplicon as observed by gel electrophoresis (appendix I). The amplification efficiencies of cDNA at different concentrations level of RNA were greater than 95% and identical to the respective standards used. Melt curve analyses of the amplicons using cDNA showed the predicted melting profiles and all amplicons were validated by DNA sequencing.

Using these assays, NCAM, RET and GFRα2 but not GFRα1, were detected in BE(2)-C cells (Fig. 3.1A). In BE(2)-C cells, GFRα1 transcript level was below the detection limit of the assay and estimated to be less than $1:10^6$ when expressed as the ratio of GFRα1 to GAPDH. Gel electrophoresis of the PCR products further confirmed the expressions of GFRα2, RET, and NCAM, and the absence of GFRα1 in BE(2)-C cells (Fig. 3.1B). The significant expressions of GFRα2, NCAM and RET in BE(2)-C cells thus provided a suitable model for further studies.

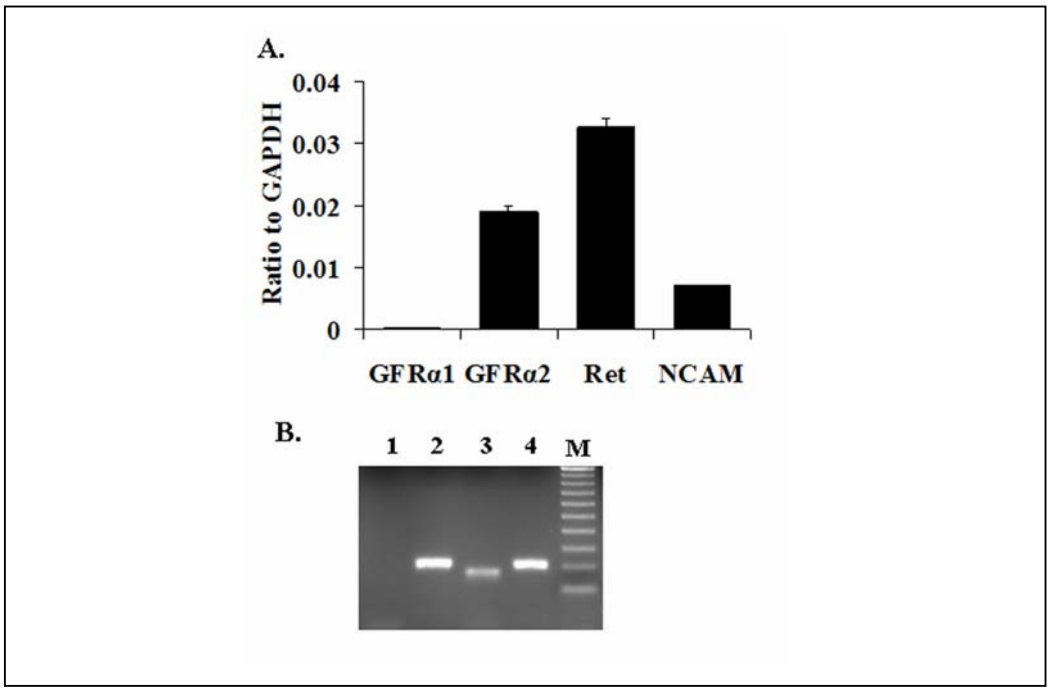

Figure 3.1. Expression levels of GFRα, RET and NCAM transcripts in human neuroblastoma BE(2)-C cells measured by quantitative real time PCR. A, GFRα2, RET and NCAM were expressed at significant levels when compared to GFRα1. The expression of GFRα1 was below the detection limit of the assay (< $1:10^6$, when expressed as the ratio of GFRα1 to GAPDH). **B**, Amplification of GFRα1 (lane 1), GFRα2 (lane 2), RET (lane 3) and NCAM (lane 4) from BE(2)-C cells using primers as described in the Material and Methods. No visible band was observed with control samples either with a single primer or the absence of the template (data not shown). Loading marker shown, M, marker 100-1000bp, with increament of 100bp each band. The results were expressed as mean ± S.E.M. of at least three independent experiments.

3.2.2 Regulation of MAPK (ERK1/2) phosphorylation by GDNF and NTN

Both GDNF and NTN activated MAPK (ERK1/2) rapidly in BE(2)-C cells (Fig. 3.2A). The responses to GDNF and NTN were similar in kinetics and sustainable over a period of six hours (Fig. 3.2B). The MEK1/2 inhibitor, U0126, inhibited GDNF and NTN induced phosphorylation of MAPK (ERK1/2) in a dose-dependent manner (Fig. 3.2C). At the concentration used, there was no evidence of cell deaths as measured by MTT conversion assay (data not shown). This result

suggests that GDNF and NTN activate MAPK signaling by phosphorylation on Thr202/204 of ERK1/2 through GFRα2.

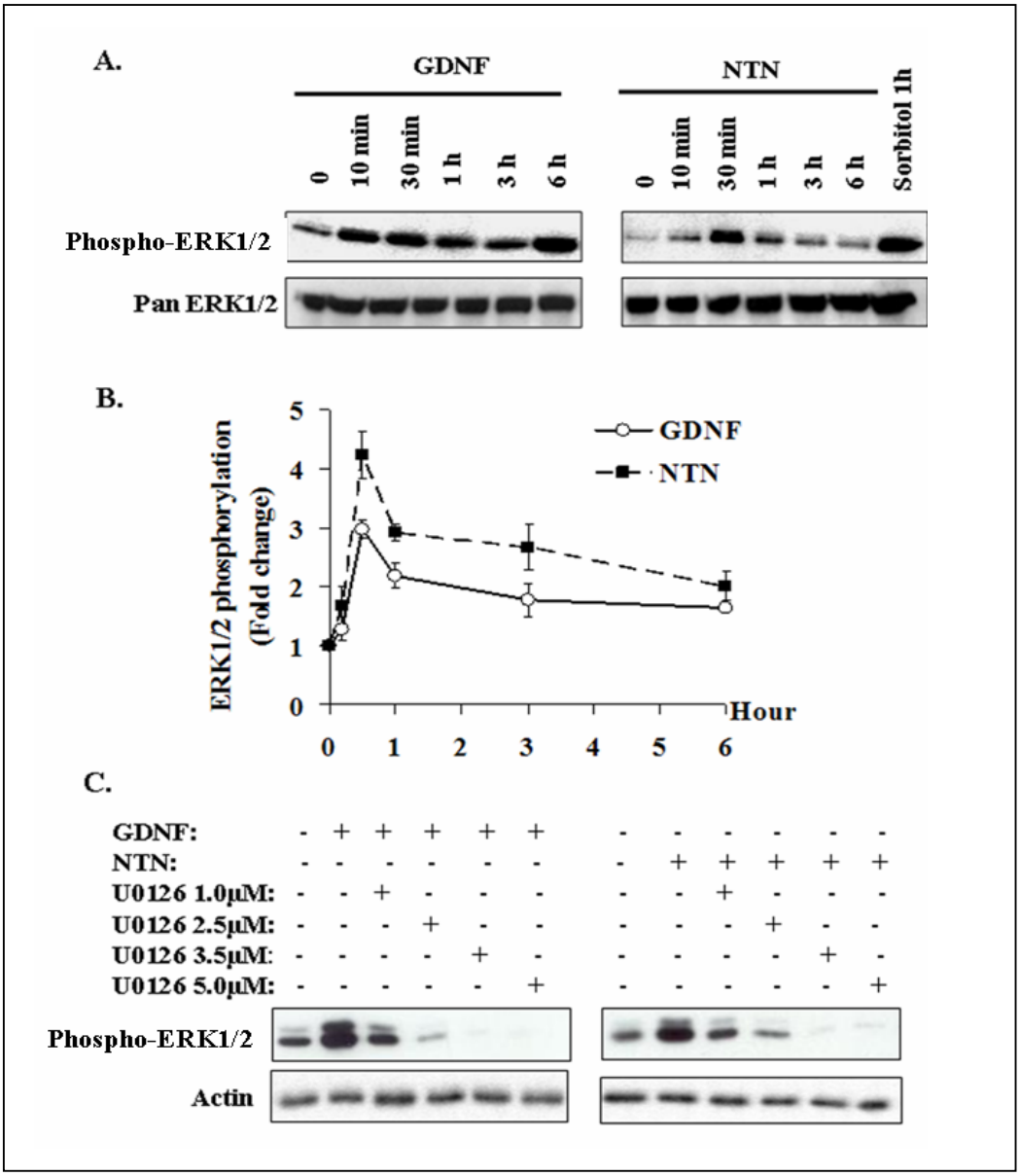

Figure 3.2. GDNF and NTN induced MAPK (ERK1/2) phosphorylation in BE(2)-C cells. A, Cells were stimulated with either GDNF or NTN and phosphorylated ERK1/2 was detected by Western Blot. **B**, Kinetic analyses of GDNF and NTN induced ERK1/2 phosphorylation. The blots were stripped and re-probed with anti-pan ERK1/2 antibody for the verification of protein loading (bottom panels). **C**, Concentration dependent inhibition of MAPK activation by U0126 in GDNF and NTN stimulated BE(2)-C cells. The cells were pretreated for 20 minutes with different concentrations of U0126 inhibitor before exposure to GDNF or NTN for a further 10 minutes. The results were expressed as standard deviations of triplicate measurements and similar results were observed for three independent experiments.

3.2.3 Regulation of miRNA precursor expressions by GDNF and NTN

Regulation of miRNAs by GDNF ligands is currently not known. Whether GDNF and NTN may regulate the expression of miRNAs in various cellular processes remains unclear. In order to address the issue of ligands receptor specificity, the human BE(2)-C cells, which express GFRα2 but not GFRα1, were used to examine the regulation of some miRNA precursors (pre-miRNAs and pri-miRNAs) by GDNF and NTN.

A total of 23 pairs of pre-validated primers designed to anneal to the hairpin of miRNA precursors (Schmittgen *et al.*, 2004) were used to quantify cDNA samples prepared from BE(2)-C cells. Initial attempts to co-reverse, transcribe and accurately quantify both U6 and the miRNA precursors simultaneously were unsuccessful. The amplification of U6 from cDNA samples prepared from 1 μg of RNA consistently show Ct values of about 14 cycles (Fig. 3.3A). This is equivalent to the amplification of 10^7 copies of GFRα2 which has a similar amplicon size and PCR efficiency (3.5 cycles per log dilution). The failure to detect amplicon after 40 cycles (detection limit equivalent to one copy per reaction) defines the expression levels of the miRNA precursors as undetectable. BE(2)-C was found to express eight of the twenty-three distinct miRNA precursors (miR-16, miR-18, miR-21, miR-24-2, miR-92-1, miR-93-1, miR-107 and miR-124a-2). All amplicons showed distinct melt curves (Fig. 3.3B) and the sizes were verified by gel electrophoresis (Fig. 3.3C). GDNF was found to transiently up-regulate the expressions of miR-21 and miR-24-2 precursors significantly (Fig. 3.4A). Interestingly, NTN was found to down-regulate the expression of miR-92-1 precursor (Fig. 3.4B). No significant changes in the expression of the other miRNA precursors were observed.

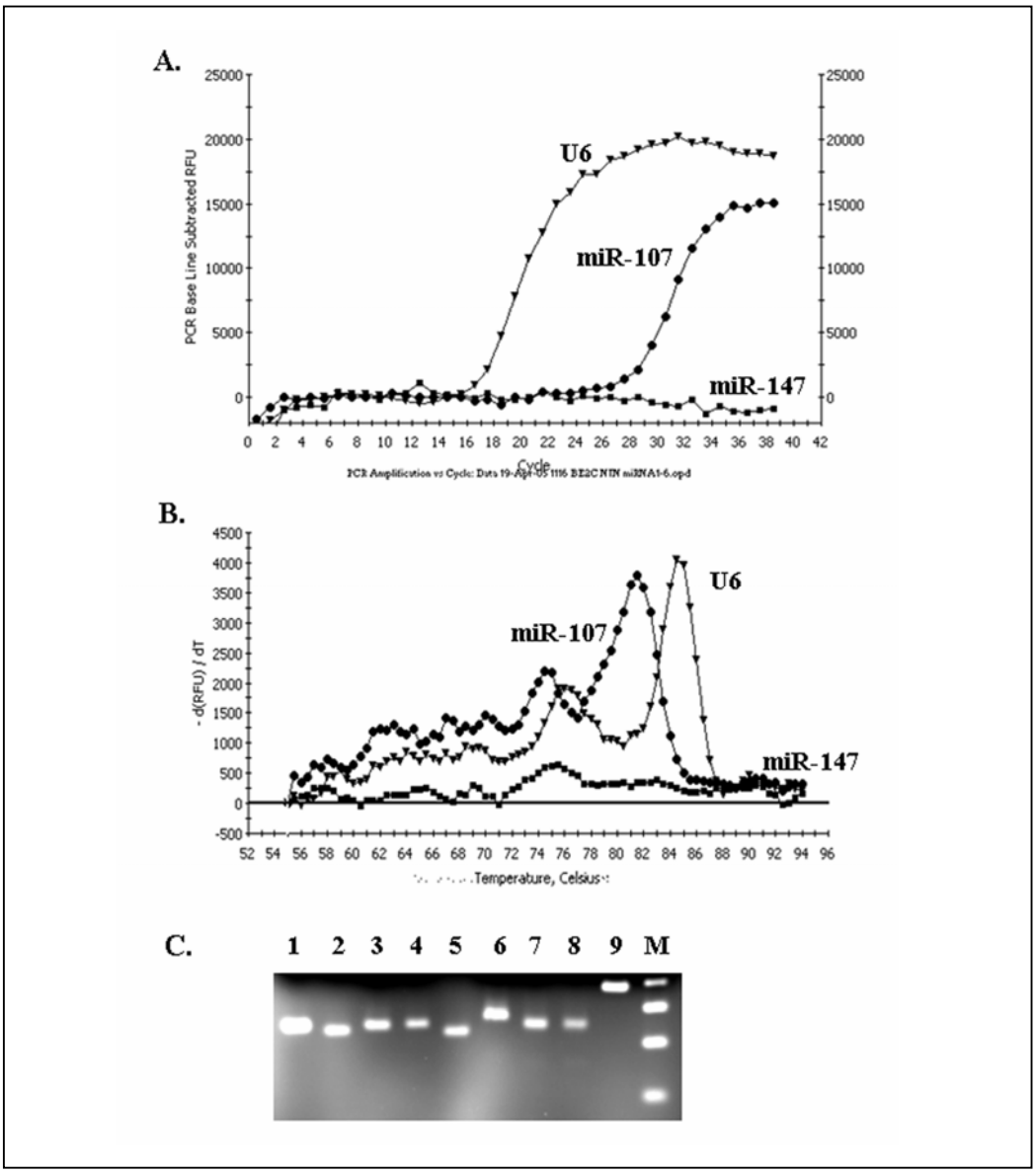

Figure 3.3. Real time PCR amplifications of miRNA precursors. Gene specific primers designed to the hairpin of miR-107 and miR-147 precursors were used for amplification using cDNA samples prepared from BE(2)-C cells. **A**, Real time PCR quantification plot showing amplifications of U6 and miR-107. miR-147 amplicon was not detected, even after 40 cycles of amplification. No template controls showed background fluorescence, even after 40 cycles of amplification. **B**, Melt curve analyses after 40 cycles of amplification. Melt curve analyses after amplifications showed distinct peaks of miR-107 and U6 products. **C**, Gel electrophoresis of short hairpin products after amplification by real time PCR. Amplifications were carried out using primers for the precursors of miR-107 (lane 1), miR-124a-2 (lane 2), miR-92-1 (lane 3), miR-93-1 (lane 4), miR-21 (lane 5), miR-24-2 (lane 6), miR-16 (lane 7), miR-18 (lane 8) and U6 (lane 9). The amplified products and the 25 bp DNA marker, with increment of 25 bp each band (M) were resolved in a 4% agarose gel. Similar results were obtained for at least three independent experiments.

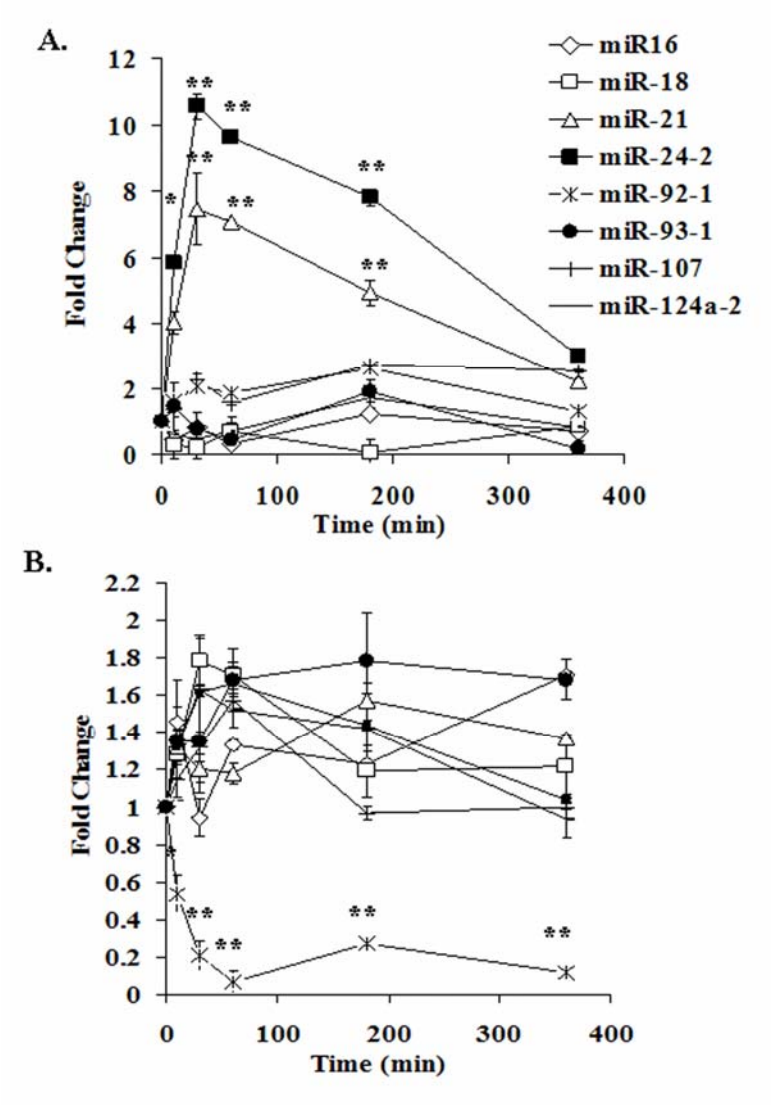

Figure 3.4. Regulation of miRNA precursors expressions by GDNF and NTN. miRNA precursors expression levels in BE(2)-C cells were expressed as fold changes on stimulation with GDNF (**A**) and NTN (**B**) over a period of six hours. Eight distinct miRNAs precursors were detected in BE(2)-C cells. Similar results were obtained for at least three independent experiments. Error bars indicate standard deviations of triplicate measurements. Significant differences in gene expression between ligand stimulated and control samples were calculated using paired Student's t-test. A value of $P < 0.05$ was considered significant (**$P < 0.001$, *$P < 0.05$).

To determine the contribution of MAPK pathway in the regulation of the expression of miRNA precursors, U0126 was used to inhibit MEK1/2 activation (Fig. 3.5). At the sub-maximal dose (2.5 μM), U0126 inhibited the up-regulation of miR-21 (Fig. 3.5A) and miR-24-2 (Fig. 3.5B) precursor expressions induced by GDNF, and the down-regulation of miR-92-1 precursor expression by NTN (Fig. 3.5C).

3.2.4 Differentiation of BE(2)-C cells with GDNF and NTN

Both miR-21 and miR-24-2 have previously been shown to be up-regulated in TPA differentiated HL-60 (Kasashima *et al.*, 2004) and retinoic acid induced differentiation of embryonic stem cells (Houbaviy *et al.*, 2003). As these miRNA precursors were similarly up-regulated by GDNF in BE(2)-C cells (Fig. 4.4A), the morphology of BE(2)-C cells when induced by GDNF and NTN was examined over a period of five days. Morphological differentiation of BE(2)-C cells was induced by retinoic acid but not GDNF or NTN (Fig. 3.6A). The expression levels of miR-21 and miR-24-2 precursors in BE(2)-C cells were significantly increased by retinoic acid (Fig. 3.6B). Interestingly, miR-92-1, which was down-regulated by NTN, was up-regulated by retinoic acid instead (Fig. 3.6B).

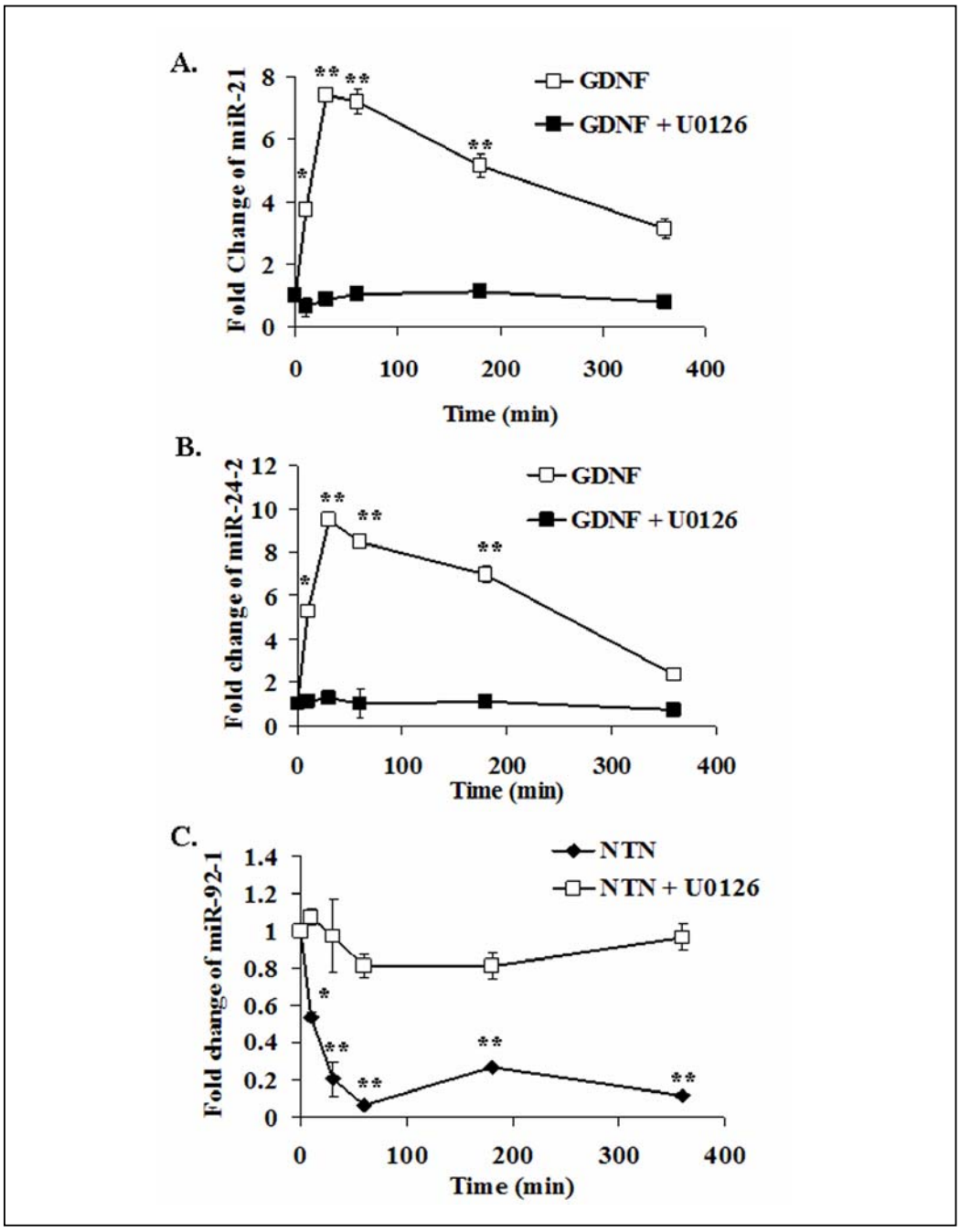

Figure 3.5. Inhibition of miRNA precursor expressions by U1026 in ligand stimulated cells. Cells were pretreated for 20 minutes with 2.5 μM of U0126 before exposure to GDNF or NTN. The up-regulation of miR-21 (**A**) and miR-24-2 (**B**) precursor expressions by GDNF was abolished in the presence of U0126. Similarly, the down-regulation of miR-92-1 by NTN (**C**) was abolished by U0126. The results were reproduced in at least three independent experiments. Error bars indicate standard deviations of triplicate measurements. Significant differences in the expression of the genes between ligand stimulated and control samples were calculated using paired Student's t-test. A value of $P < 0.05$ was considered significant (**$P < 0.001$, *$P < 0.05$).

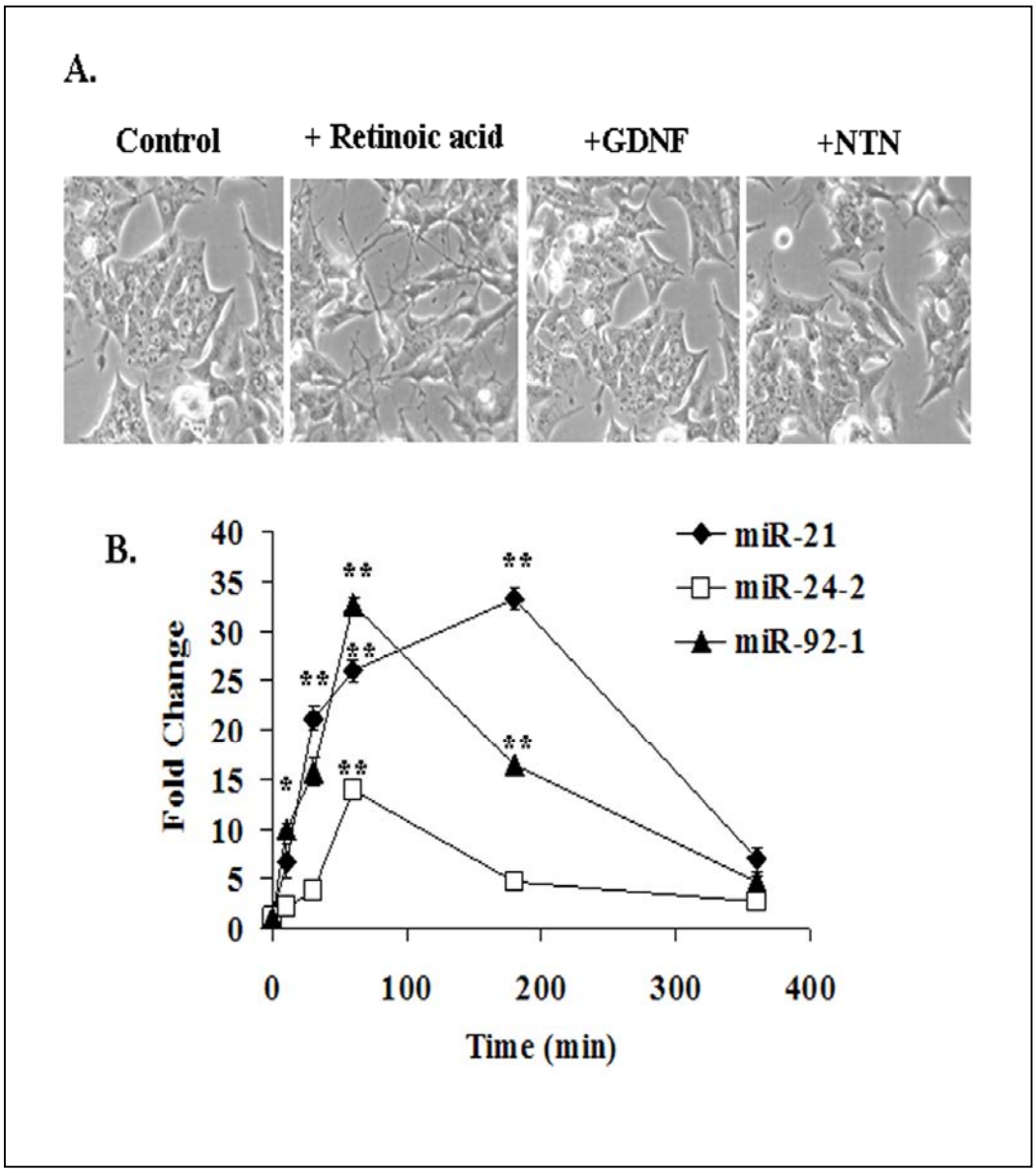

Figure 3.6. Retinoic acid-induced differentiation of BE(2)-C cells. A, Treatments of BE(2)-C cells with retinoic acid, GDNF or NTN. Cells (20,000) were seeded on six well plates overnight in DMEM supplemented with 10% FBS. Cells were then incubated in 0.5% FBS supplemented media, with or without all-trans retinoic acid (5 μM), GDNF (50 ng/ml) or NTN (50 ng/ml), and were incubated for three days. Retinoic acid treated cells showed neurite extension but not GDNF or NTN. (magnification x200) The experiment was repeated at least three times with similar results. **B,** Regulation of miRNA precursors expressions in BE(2)-C by retinoic acid. The expressions of miR-21, miR-24-2 and miR-92-1 precursors were up-regulated by retinoic acid over a period of six hours. Similar results were obtained for at least three independent experiments. Error bars indicate standard deviations of triplicate measurements. Significant differences in expression of miRNA precursors between ligand stimulated and control samples were calculated using paired Student's t-test. A value of $P < 0.05$ was considered significant (**$P < 0.001$, *$P < 0.05$).

3.3 Discussion

The results from this study reveal a novel function of GFRα2 in the regulation of miRNA precursors. This function is mediated by MAPK (ERK1/2) and the downstream regulation of distinct miRNA precursors is dependent on the ligand used.

GFRα2 and GFRα1 share about 48% amino acid identity, and the nearly complete conservation of cysteine residues suggests that these two receptors have similar three-dimensional structures and share similar functions (Jing *et al.*, 1997; Scott and Ibanez, 2001). This is consistent with evidence from transfected cells and primary cultures where GDNF and NTN have similar properties in activating the multi-component receptor complex (Airaksinen *et al.*, 1999; Baloh *et al.*, 1997; Charlet-Berguerand *et al.*, 2004; Coulpier *et al.*, 2002; Scott and Ibanez, 2001; Wang *et al.*, 2000). Furthermore, midbrain dopaminergic neurons which only express GFRα1 appear to survive equally well with both GDNF and NTN *in vitro* and *in vivo* (Horger *et al.*, 1998), and respond to neither ligands in the absence of GFRα1 (Cacalano *et al.*, 1998). However, there are observations of distinct functional differences with the use of specific ligands. Although GDNF and NTN promote the survival of dopaminergic neurons through GFRα1 (Akerud *et al.*, 1999; Cacalano *et al.*, 1998), only GDNF possesses neuritogenic and hypertrophic effects (Akerud *et al.*, 1999). In cultured sympathetic neurons, GDNF is able to promote the survival of culture sympathetic neurons through GFRα2 but NTN is unable to promote survival through GFRα1 (Buj-Bello *et al.*, 1997). Furthermore, GDNF but not NTN, is able to promote the axonal growth of DRG neurons through GFRα1 (Paveliev *et al.*, 2004). The emerging view is that the crosstalk of exogenously applied GDNF and NTN with the non-preferred receptors may result in distinct functions. Concurrent with this

study, Lee et al have recently shown that GDNF and NTN have distinct and differential biochemical effects on cells expressing only GFRα1 (Lee *et al.*, 2005b).

In order to address the significance of GDNF and NTN crosstalk in regulating the expression of miRNA in a defined system, a cell that expresses the multi-component receptor complex with only one particular GFRα is required. The distinct advantage of using quantitative real time PCR over conventional end-point PCR-based assays is that it allows the reliable quantitative definitions of specificity, sensitivity and efficiency (Wong and Medrano, 2005). Using the highly specific, efficient and sensitive quantitative real time PCR assays developed in this study, BE(2)-C cells are found to express NCAM, RET and GFRα2, but not GFRα1. The presence of GFRα2 but not GFRα1 in BE(2)-C cells is consistent with the previous observation (Kobori *et al.*, 2004) but is not so in a recent report using semi-quantitative PCR (Hansford and Marshall, 2005). Consistent with the suggestion that both GDNF and NTN can activate the same multi-component receptor system, GDNF has been shown to induce the enhancement of phosphorylation and enzymatic activity of tyrosine hydroxylase through the activation of GFRα2 (Kobori *et al.*, 2004).

GDNF and NTN are known to similarly activate a number of signaling pathways, including the extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) (Ichihara *et al.*, 2004; Pezeshki *et al.*, 2003; Takahashi, 2001; Trupp *et al.*, 1999), and regulate the expressions of various immediate early response genes (Fukuda *et al.*, 2003; Pezeshki *et al.*, 2003). Intriguingly, stimulation with GDNF and NTN resulted in similar kinetics of activation of ERK1/2 but regulated the expressions of distinct miRNAs. The rapid changes in gene expression of miR-21, miR-24-2 and miR-92-1 paralleled that of the rapid induction of the early response

genes (Murphy *et al.*, 2004; Sng *et al.*, 2004). Interestingly, miR-92-1 which was rapidly down-regulated by NTN remained unaffected by GDNF stimulation, but was instead found to be up-regulated by retinoic acid stimulation. Recently, neurotrophins have been shown to regulate the expression of a miRNA (miR-132) through a cAMP dependent pathway, resulting in changes in neuronal morphology (Vo *et al.*, 2005). The stimulation of GFRα2 by either GDNF or NTN did not result in neurite outgrowth in BE(2)-C cells. However, when stimulated by retinoic acid, these cells showed extensive neurite outgrowth and up regulations of miR-21, miR-24-2 and miR-92-1.

Analyses of the proximal sequences of miR-21, miR-24-2 and miR-92-1 by computational prediction of eukaryotic promoters (Scherf *et al.*, 2000) reveal the existence of multiple regulatory elements. This suggests that the expressions of these miRNAs may be regulated by multiple pathways similar to other RNA polymerase II mediated transcripts (Lee *et al.*, 2004; Sng *et al.*, 2004). The binding of NGF to TrkA receptor was known to activate two or more distinct signaling pathways and the inhibition of a single pathway can affect the expressions of the transcription of some genes (Marek *et al.*, 2004). It is likely that the differential regulations of distinct miRNAs expressions by GDNF and NTN in BE(2)-C cells may similarly require the concerted signaling of multiple signaling pathways. The integration of signaling pathways regulating the expression of these miRNAs may provide a means for a more precise transcriptional control depending on whether one or more pathways are activated. With the myriad of distinct signaling pathways induced by activated RET (Ichihara *et al.*, 2004; Takahashi, 2001), GDNF and NTN acting through the same receptor complex appear to activate two or more signaling mechanisms. Such an

integration of these pathways is a subject for further investigation (depicted in Fig. 3.7).

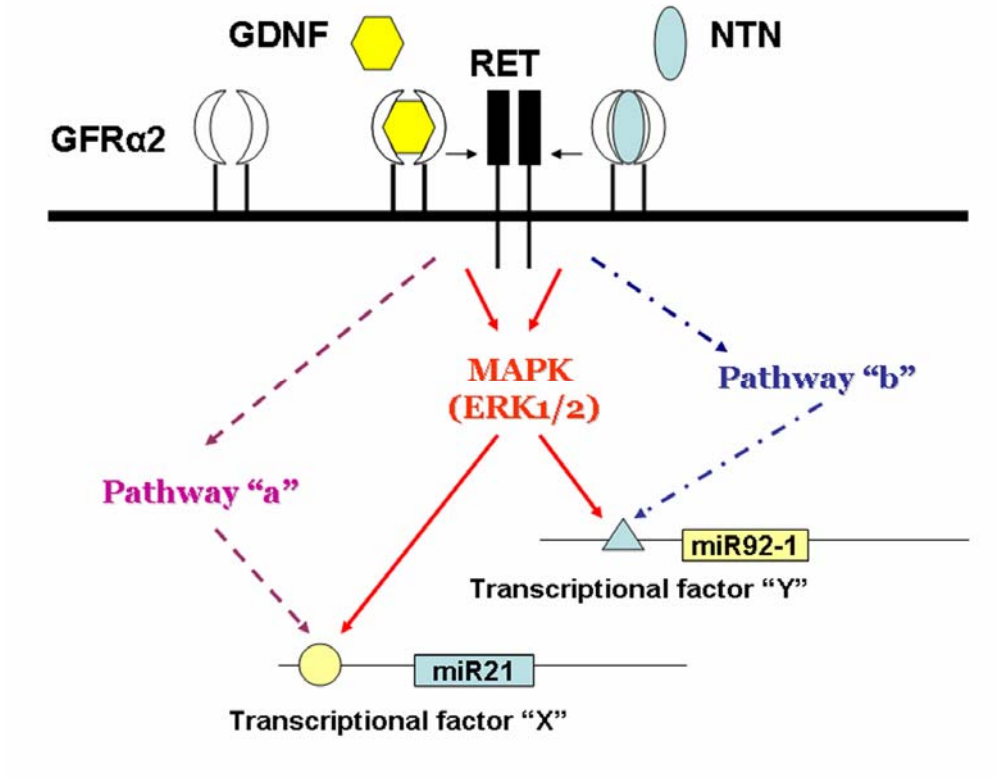

Figure 3.7. Proposed model for multiple pathways required for selection and activation of specific transcriptional factors in regulation of microRNA (miRNA) precursors expression. MAPK was activated upon binding of GDNF or NTN to GFRα2 and co-receptor RET. GDNF and NTN may differently activate another pathway upon binding to GFRα2 (Pathway "a" for GDNF, and Pathway "b" for NTN). GDNF activations of MAPK together with Pathway "a" may be required for the activation of transcriptional factor/s "X" that regulate/s the expression of miR21, whereas NTN activated MAPK together with Pathway "b" may be specific for the activation of transcriptional factor/s "Y" which regulate/s the expression of miR92-1. Such integration of two or more pathways differently activated by GDNF and NTN may contribute to the specificity of the regulation of miRNA expressions through GFRα2 and co-receptor RET.

miRNAs are now thought to be involved in a number of physiological and developmental processes (Croce and Calin, 2005; Harfe, 2005; Miska, 2005). Both miR-21 and miR-24-2, which were up-regulated by GDNF and retinoic acid but not NTN in BE(2)-C cells, have been demonstrated to be involved in cell proliferation and differentiation, and are over-expressed in various human cancers (Chan *et al.*, 2005; Houbaviy *et al.*, 2003; Iorio *et al.*, 2005; Kasashima *et al.*, 2004). NTN but not GDNF rapidly down-regulates miR-92 expression in BE(2)-C cells. Interestingly, retinoic acid showed an opposite up-regulation of the same miRNA. Although miR-92 has previously been shown to be amplified and over-expressed as a polycistronic miRNA cluster (Ota *et al.*, 2004a) and up-regulated in expression in some cancers (Calin *et al.*, 2004), the function of miR-92 is still to be determined. The specific regulation of the expressions of these miRNAs by GDNF and NTN suggests distinct functions associated with the activation of GFRα2. It is likely that many more miRNAs may be involved in various cellular processes and that the expressions of specific clusters of these miRNA may be cell type specific.

miRNAs are thought to exert their functions by binding to complementary sequences on target mRNAs, inducing cleavage of mRNAs or repression of protein translation (Bartel, 2004; Ke *et al.*, 2003; Nelson *et al.*, 2003; Pillai, 2005). The search for miRNA targets by various algorithms (John *et al.*, 2004; Krek *et al.*, 2005; Lewis *et al.*, 2003) has been met with some success although a recent study has shown that some targets do escape prediction (Nakamoto *et al.*, 2005). To date, the specific targets of miR-21, miR-24-2 and miR-92-1 remain unknown and predictions by various algorithms suggest more than a hundred putative targets for each of these microRNAs.

In conclusion, this study showed the differential regulation of distinct miRNA precursors by GDNF and NTN through the same GFRα2 and the multi-component receptor system. BE(2)-C cells preferentially express GFRα2a and GFRα2b isoforms (Chapter 5, Figure 5.3). It is unknown if GDNF or NTN preferentially activate one particular GFRα2 isoform or both isoforms concurrently, resulting in the expression of the distinct miRNAs. The differential effects of two related ligands acting on the same multi-component receptor system and the possibility that specific isoforms may contribute to the observed differences illustrates the complexity of the interactions of GFLs and GFRα2.

Chapter 4 Part II: Differential expressions, biochemical activities, and neuritogenic activities of the alternatively spliced GFRα2 isoforms.

4.1 Background and objectives

To date, at least 3 alternatively spliced isoforms of GFRα2 receptor have been identified in mammalian systems, namely GFRα2a (1393 bp), GFRα2b (1077 bp), and GFRα2c (993 bp) (Figure 4.1). Little is known about the biochemical and signaling property of GFRα2 receptor and the significance of GFRα2 alternative splicing. It is unknown if these isoforms serve distinct or redundant function. To gain a better insight into the biological relevance in the central nervous system, the expression levels of the isoforms in different regions of the human brain were quantified by highly specific real time PCR assays. The biological functions of the isoforms were then examined in a neuronal differentiation model using Neuro2A cells.

Cultured clonal cell lines can serve as useful experimental models for investigating cellular morphologies and biochemical events during neuronal differentiation. It has some advantages over most alternatives such as primary cultured neurons. With clonal cell cultures, the cellular states can be controlled externally by changing the compositions of the media and conditions. In addition, the morphological, biochemical and molecular biological aspects of the cells can be analyzed quantitatively as all the cells are clonal. Primary cultured neurons have a distinct limitation in that *in vivo* neurons usually bear neurites on the day of preparation and the models may represent 're-morphogenesis', such as 're-neuritogenesis' and 're-neurite outgrowth', in the culture dishes. Given the limitations, the integration of insights from both primary culture systems and clonal cell lines can provide better understandings of the general mechanisms involved in neural morphogenesis.

Neuroblastoma cells are widely used as cultured clonal cell line models for the studies of neuronal differentiation (Clagett-Dame *et al.*, 2006; Edsjo *et al.*, 2006).

Neuro2a, a mouse neuroblastoma cell line, when differentiated by a variety of agents has been shown to induce axon-like and dendrite-like processes (Wu *et al.*, 1998). Under normal growth conditions, Neuro2a cells spontaneously sprout a basal level of neurites. However, treatments with a variety of stimuli cause these cells to develop extensive neurites similar to changes observed in hippocampal and cortical cultures (Ahmari *et al.*, 2000; Washbourne *et al.*, 2002). Greater than 98% of proteins identified by quantiative tissue proteomic analyses of Neuro2a and the whole mouse brain are identical (Ishihama *et al.*, 2005). Furthermore, the transcriptomes of Neuro2a and neural stem cells during neural development and differentiation have been shown to share extensive similarities (Bulfone *et al.*, 2005). These studies suggest Neuro2A to be a suitable model for the analyses of the biological functions of the GFRα2 isoforms. Furthermore, Neuro2a cells express RET and NCAM but not GFRα1 or GFRα2 (see Figure 4.4).

Here, we show that ligands activation of the isoforms differentially activated MAPK (ERK1/2) and AKT signalings, and regulated distinct early response genes. Furthermore, both GDNF and NTN induced neurite outgrowth through GFRα2a and GFRα2c, but not GFRα2b. This study thus, provides the first piece of evidence of a distinct biochemical and neuritogenic activity of the alternatively spliced GFRα2 isoforms.

4.2 Results

4.2.1 Differential expression profiles of GFRα2 spliced variants

The expression level of GFRα2 spliced variants in specific regions of the brain is currently unknown. To address this issue, sequence independent real time PCR assays have been developed to quantify each of the spliced variants with high specificity and sensitivity.

In order to discriminate between the three spliced variants of human GFRα2, exon overlapping primers were designed across exon 1 and 2, 1 and 3 or 1 and 4. This is to enable the specific detection and quantification of GFRα2a, GFRα2b and GFRα2c, respectively (Fig 4.1).

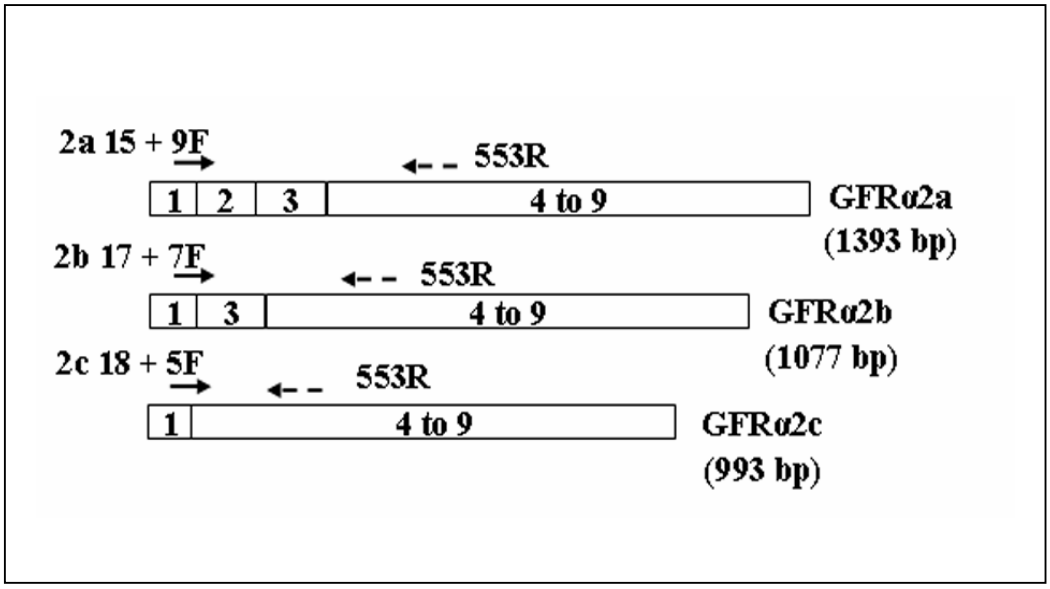

Figure 4.1. Real time PCR primers and genomic organizations of the human GFRα2 isoforms. Schematic diagram of exon organization of human GFRα2 isoforms and design of specific primer for quantitative real time PCR. A specific forward primer, [2a 15+9F] for GFRα2a, [2b 17+7F] for GFRα2b, and [2c 18+5F] for GFRα2c was designed across specific exon junction, while a common reverse primer (553R) was designed for amplification of all GFRα2 isoforms.

As the amplification products of GFRα2a (545 bp), GFRα2b (233 bp) and GFRα2c (172 bp) were different in sizes, it is critical to determine the optimal cycling parameters for the selective amplification of each of the transcript. A dwell time of 30 seconds for annealing, 60 seconds for denaturation at 95^{o}C and another 60 seconds for extension at 72^{o}C was found to be optimal for the amplifications of all three isoforms. The slopes of the plots of Ct versus $\log_{10}$ mole of the human GFRα2a, GFRα2b, and GFRα2c standards were 3.37 ± 0.30 (r^2 = 0.98), 4.12 ± 0.41 (r^2 = 0.99), and 3.82 ± 0.54 (r^2 = 0.99) respectively (Fig. 4.2). The detection limits of the assays were estimated to be fewer than 100 copies of transcripts per reaction. The samples were diluted in parallel with the standards. The specificity of amplifying a particular isoform when compared to the other variants was greater than 10^6 fold (Fig. 4.2). Hence, when using GFRα2a exon overlapping primers, the amplifications of GFRα2b and GFRα2c were at least 10^6 fold less efficient than amplifying GFRα2a.

Using these highly sensitive and specific assays, the expression levels of the GFRα2 alternatively spliced isoforms were quantified in the caudate nucleus, cortex, putamen, substantia nigra, sub-thalamic nucleus and thalamus of the human brain (Fig. 4.3). The three GFRα2 isoforms were detected at significant levels (> 10^4 copies/reaction) in all areas of the brain, with expression levels highest in the cortex. In the cortex, all three isoforms were expressed at comparable levels, with GFRα2b expression significantly lower than GFRα2c (P<0.01).

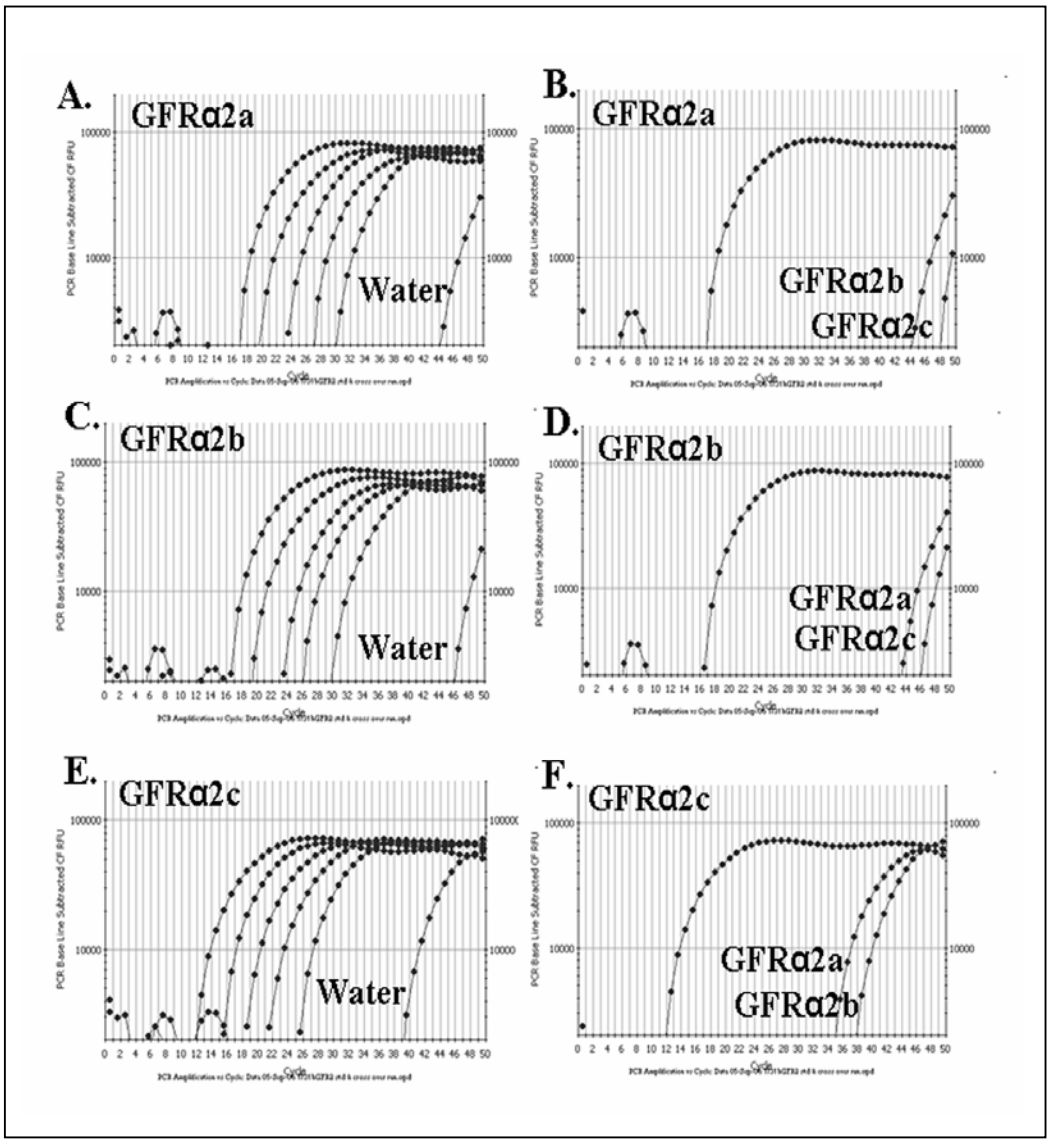

Figure 4.2. Quantitative real time PCR assay for human GFRα2 isoforms. Amplifications of five $\log_{10}$ dilutions of GFRα2a (**A**), GFRα2b (**C**), and GFRα2c (**E**), plasmids standards. Negative controls with no templates (Water) were carried out simultaneously. The slopes of the plots of Ct versus $-\log_{10}$ mol of GFRα2a, GFRα2b and GFRα2c were 3.37 ± 0.30 (r^2 = 0.98), 4.12 ± 0.41 (r^2 = 0.99), and 3.82 ± 0.54 (r^2 = 0.99), respectively. Equal quantities (6 attomol) of GFRα2 isoforms were amplified separately using GFRα2a (**B**), GFRα2b (**D**) and GFRα2c (**F**) exon specific primers. The experiments were repeated at least four times with similar results.

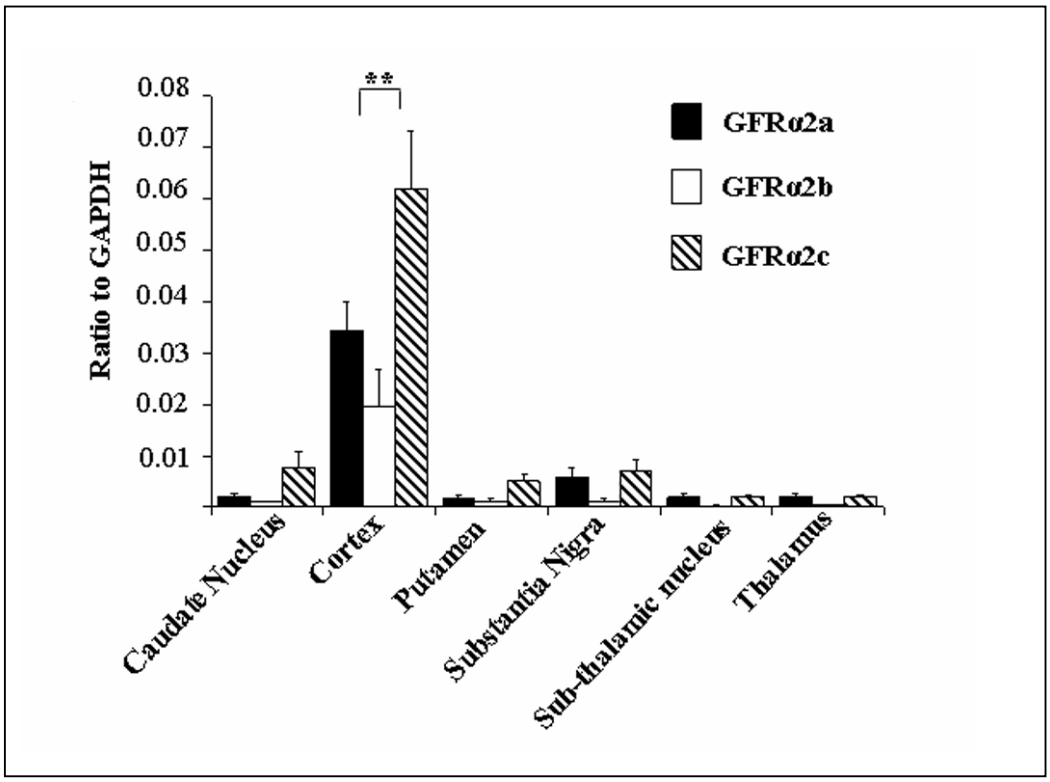

Figure 4.3. **Real time PCR quantifications of GFRα2 isoforms expression in different human brain regions.** Expression levels of human GFRα2 isoforms in different human brain regions were determined using specific primers designed for GFRα2 isoforms. The expression levels were normalized to the levels of GAPDH in the same tissue. The results were expressed as mean ± S.E.M. (n=3). Significant differences in percentage of differences between expressions of isoforms were calculated using paired Students t-test. A value of $P<0.05$ was considered significant (**$P<0.001$).

4.2.2 Establishment of Neuro2A cell models stably expressing GFRα2 isoforms.

To investigate the biological significance of alternatively spliced GFRα2 isoforms, stable transfectants were generated using Neuro2a cells. Neuro2a cells express Ret and NCAM endogenously, but not GFRα1 or GFRα2 receptors (Fig. 4.4). The expression levels of GFRα2 isoforms in stably transfected Neuro2a cells (Fig. 4.4) were comparable to those expressed in the human cortex (Fig. 4.3).

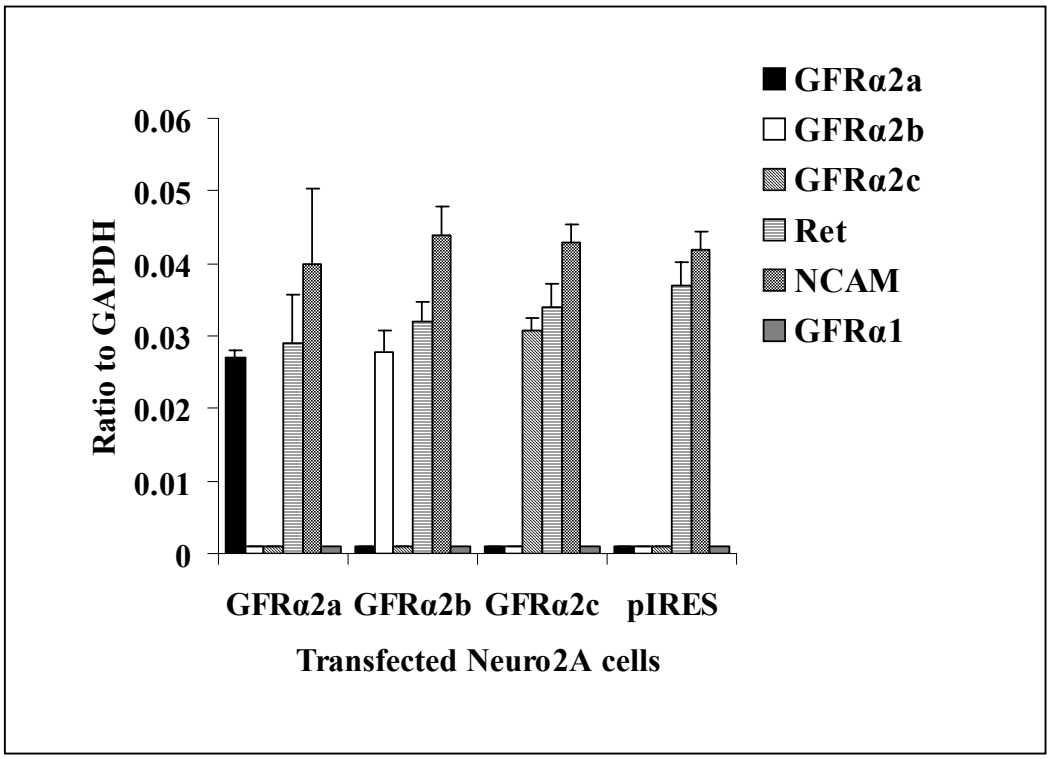

Figure 4.4. Establishment of Neuro2A cells models stably expressing GFRα2 isoform. Expression levels of GFRα2 isoforms in Neuro2A transfected with different GFRα2 isoforms were quantified using quantitative real time PCR, with specific primers. Cells transfected with vehicle control pIRES showed no expression of any GFRα2 isoforms and GFRα1 receptor, while each transfected cell line expressed significant levels of one GFRα2 isoforms. Endogenous expression levels of Ret and NCAM were comparable in all GFRα2 isoforms transfected cell lines.

4.2.3 GFRα2 isoforms differentially activated ERK1/2 and Akt

Using the Neuro2A cells stably expressing GFRα2 isoforms, the ligand induced signaling mechanisms of GFRα2 isoforms were investigated. When stimulated with NTN, all the three isoforms induced the rapid phosphorylation of ERK1/2 (Fig. 4.5). However, when stimulated with GDNF, GFRα2a and GFRα2c but not GFRα2b (Fig. 4.5) induced significant increase of ERK1/2 phosphorylation (> 2 fold). The kinetic and fold change of ERK1/2 phosphorylation was further elucidated using a dot-blot assay (Fig. 4.6A-C). The extent of ERK1/2 phosphorylation was similar when GFRα2a (Fig. 4.6A) and GFRα2c (Fig. 4.6C) were activated with either GDNF or

NTN. However, GFRα2b showed rapid and significant phosphorylation of ERK1/2 only with NTN stimulation but not by GDNF (Fig. 4.6B). Both GDNF and NTN induced ERK1/2 phosphorylation in a dose response manner in GFRα2a (Fig. 4.6D) and GFRα2c (Fig. 4.6F) transfectants. As compared to the stimulation with NTN, GFRα2b, when stimulated with GDNF, showed no significant increase in ERK1/2 phosphorylation even at the highest dose (Fig. 4.6E). No significant increase in the phosphorylation of ERK1/2 was observed in vector (pIRESneo) control transfected Neuro2A cells when stimulated with either GDNF or NTN (data not shown).

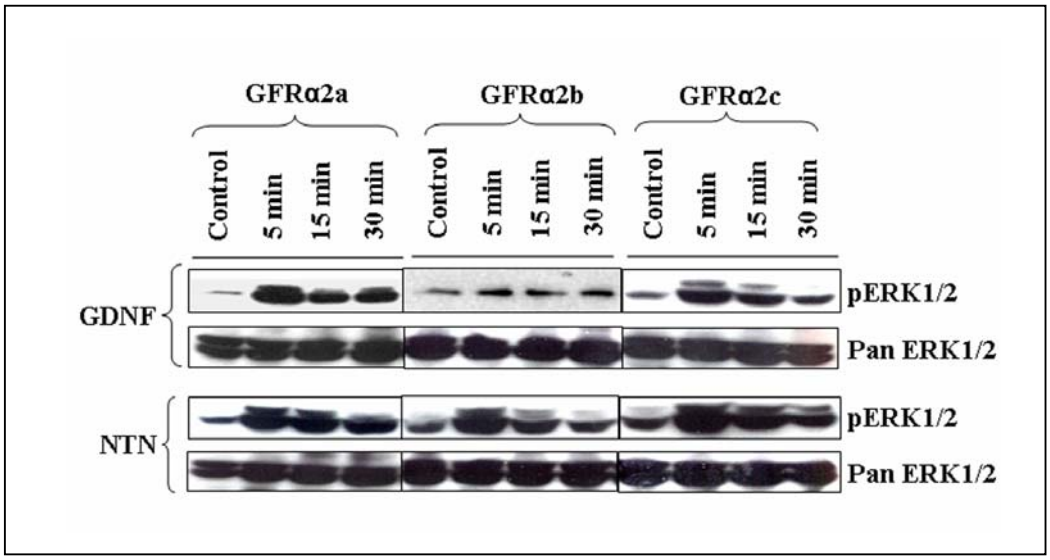

Figure 4.5. Ligand stimulated ERK1/2 activations in GFRα2 isoform transfected Neuro2A cells. Cells were stimulated in serum free media, with or without GDNF or NTN (50 ng/ ml) for the time indicated. Five micrograms of protein were loaded and separated by SDS electrophoresis, phosphorylated ERK1/2 was detected by Western Blot. Blots were stripped and reprobed with pan antibody for loading control. Experiments were repeated three times with two individual clones, showing similar results.

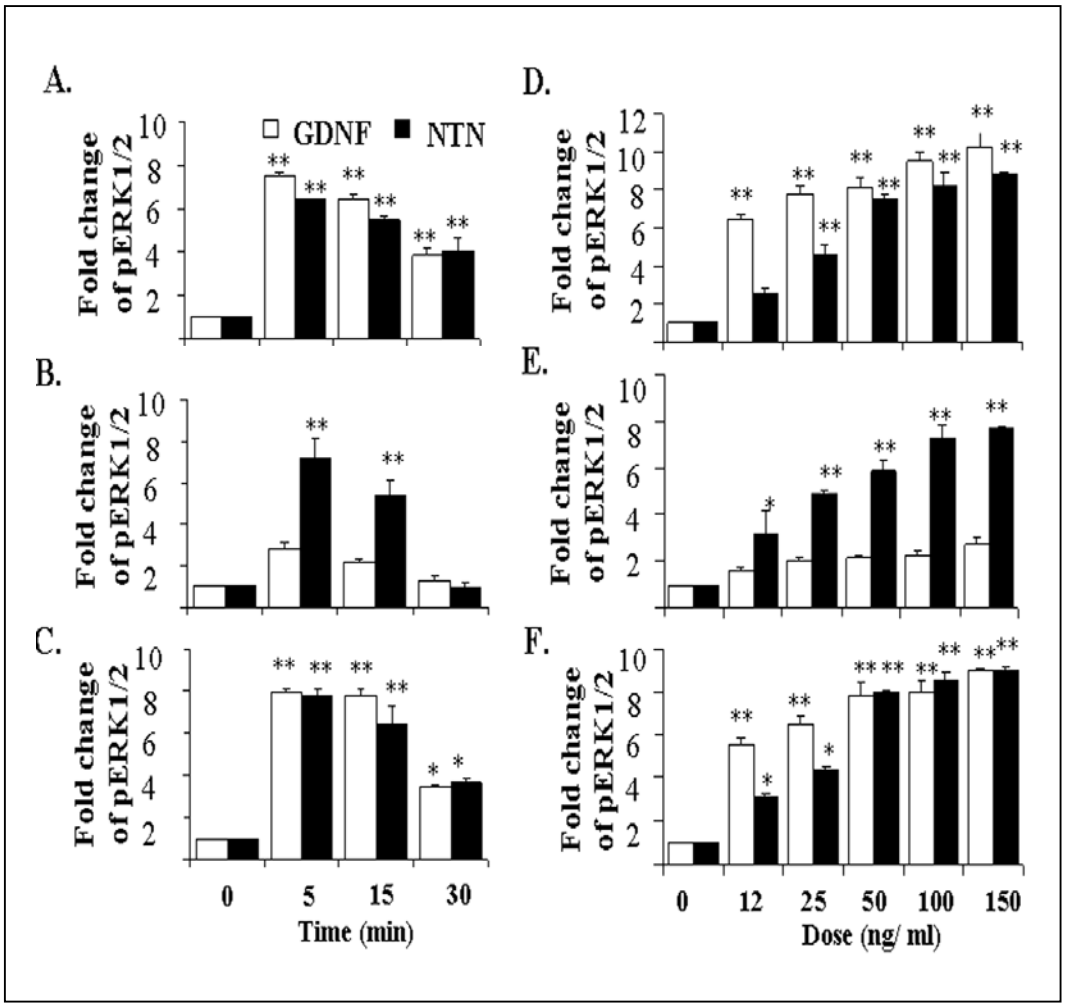

Figure 4.6. Kinetic analysis and dose response of GDNF and NTN regulation of ERK1/2 activation in GFRα2 isoform transfected Neuro2A cells. A-C, Kinetic of GDNF and NTN induced ERK1/2 activations in GFRα2a (**A**), GFRα2b (**B**) and GFRα2c (**C**). Cells were treated with or without 50 ng/ml of GDNF or NTN for time indicated. **D-E,** Dose response of GDNF and NTN induced ERK1/2 activations in GFRα2a (**D**), GFRα2b (**E**) and GFRα2c (**F**) isoforms. Cells were treated for 10 minutes with ligand, of dose indicated. For these studies, 5 μg of protein were loaded per well for dot blot assay and immuno-blotted for phospho-ERK1/2. Standard deviations were shown for triplicate wells. Experiments were repeated three times for two individual clones with similar results. Significant differences in fold change of phosphorylated EKR1/2 between ligand stimulated and control samples were calculated using paired Students t-test. A value of $P<0.05$ was considered significant (**$P<0.001$, *$P<0.05$).

Next, the ligand regulated phosphorylation of Akt using GDNF or NTN was investigated (Fig. 4.7). NTN induced rapid and significant phosphorylation of Akt in all three isoform transfectants. However, GDNF induced the rapid and significant phosphorylation of Akt in cells expressing GFRα2b and GFRα2c, but not in GFRα2a.

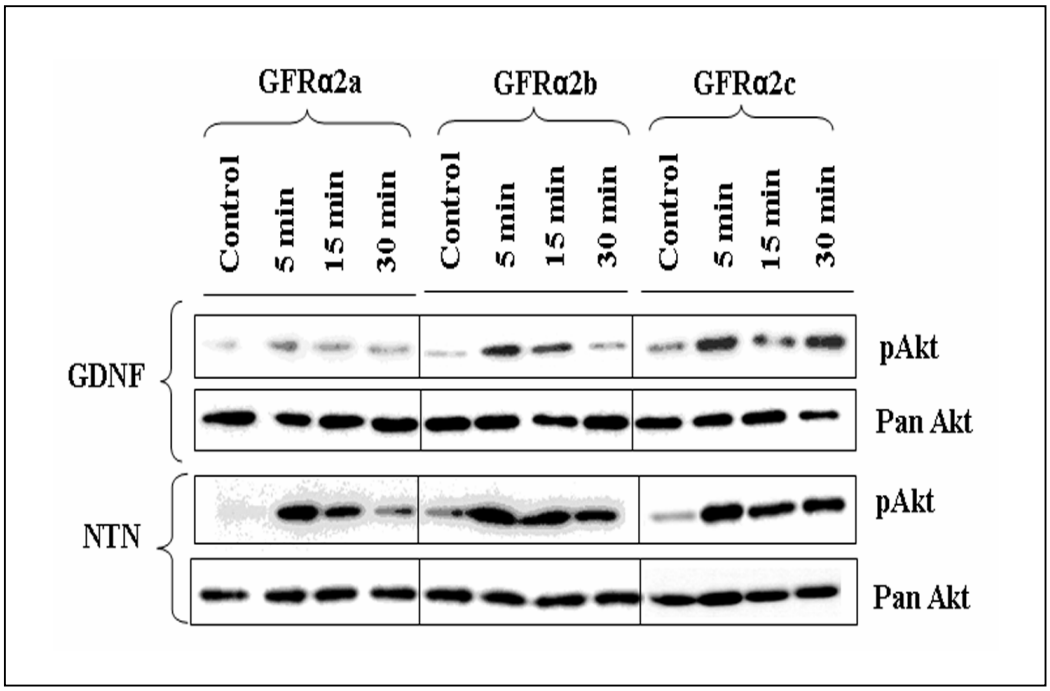

Figure 4.7. Ligand stimulated Akt activations in GFRα2 isoform transfected Neuro2A cells. Cells were stimulated in serum free media, with or without GDNF or NTN (50 ng/ml) for time indicated. Five micrograms of protein were loaded and phosphorylated Akt was detected by Western Blot. Blots were stripped and reprobed with pan antibody for loading control. Experiments were repeated three times for two individual clones, with similar results.

The other GDNF family of ligands (GFLs), Artemin and Perserphin, did not induce significant phosphorylation of ERK1/2 or Akt in any of the GFRα2 isoform transfectants (data not shown). Additionally, neither GDNF nor NTN was found to activate p38 and JNK in any of the GFRα2 isoform transfectants, even at concentration levels as high as 100 ng/ml and over one hour of ligand stimulations (data not shown).

4.2.4 [^{125}I]GDNF bound equally well to all three GFRα2 isoforms

As GDNF failed to induce a significant increase in the phosphorylation of ERK1/2 in GFRα2b transfectant (Fig 4.5, 4.6), it is possible that GDNF may not bind to this isoform. In order to address this possibility, a ligand displacement study using [^{125}I]GDNF was conducted. GDNF displaced the binding of [^{125}I]GDNF to the three GFRα2 isoforms with similar potencies (Fig. 4.8). The IC_{50} for the displacements of cells transfected with GFRα2a, GFRα2b and GFRα2c were 3.27+/-0.02, 2.79 +/-0.16 and 2.31+/-0.09 (Mean +/- SD), respectively. Parental Neuro2A or cells transfected with pIRESneo showed no significant binding to [^{125}I]GDNF (data not shown). This result indicates that GDNF binds to all three isoforms with similar affinities.

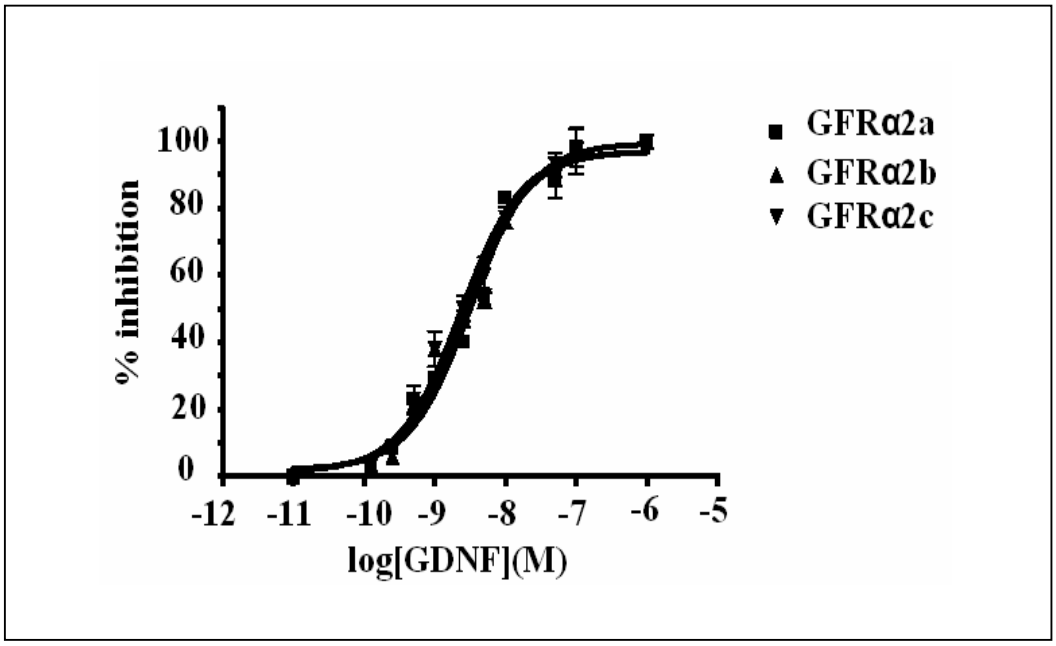

Figure 4.8. Displacement of [^{125}I]GDNF by unlabeled GDNF in GFRα2 isoform transfected Neuro2A cells. Transfectants were incubated with 50 pM of [^{125}I]GDNF in the absence or presence of increasing concentrations of unlabeled GDNF at 4˚C for four hours. The unbound ligand was removed at the end of incubation and the radioactivity associated with the cells was determined. The percentage of inhibition was expressed as mean +/- SD (n = 3) of the amount of cells associated radioactivity in presence over the absence of unlabeled GDNF. Similar results were obtained for four independent experiments.

4.2.5 GFRα2 isoforms activated different transcriptional genes

The differential activations of ERK1/2 and Akt (Fig. 4.5, 4.6, 4.7) suggest the possibility that downstream biochemical mechanisms of GFRα2 isoforms may differ. To explore this issue, the regulation of early response genes in ligand induced activation of GFRα2 isoforms was measured. Early response or immediate early genes are classes of genes that are rapidly and transiently activated in response to intracellular signaling cascades. They encode for inducible transcription factors, including the Fos, Jun and Egr protein family (Sng *et al.*, 2004). However, regulation of these genes in GFRα2 by GDNF or NTN stimulation is seldom reported. In this study, the changes in gene expression of the *fos* family (*c-fos, fosB*), *jun* family (*c-jun, jun-b*), *egr* family (*egr*1-4) and GDNF inducible transcription factors, *mGIF* and *mGZF1* in response to GDNF and NTN were measured. These factors have previously been shown to be activated with GDNF or NTN (Fukuda *et al.*, 2003; Kozlowski *et al.*, 2000; Pezeshki *et al.*, 2003; Trupp *et al.*, 1999; Yajima *et al.*, 1997).

The kinetics of gene activation over a period of six hours were quantified by real time PCR (Fig. 4.9). Distinct ligand induced early response gene expressions were observed with the activation of the different GFRα2 isoforms. GFRα2a, when stimulated by GDNF or NTN, up-regulated *egr-1* by as much as four to five folds (Fig. 4.9A, B). GFRα2b, when stimulated by GDNF or NTN, up-regulated *fosB* by greater than ten folds, when compared to the control (Fig. 4.9C, D). When stimulated with GDNF or NTN, GFRα2c up-regulated the expressions of *egr-1* and *egr-2* (Fig. 4.9E, F). With the other genes, no significant changes were observed with GDNF or NTN stimulations (data not shown). These results show that GFRα2 isoforms when stimulated induced distinct transcription of specific sets of early response genes.

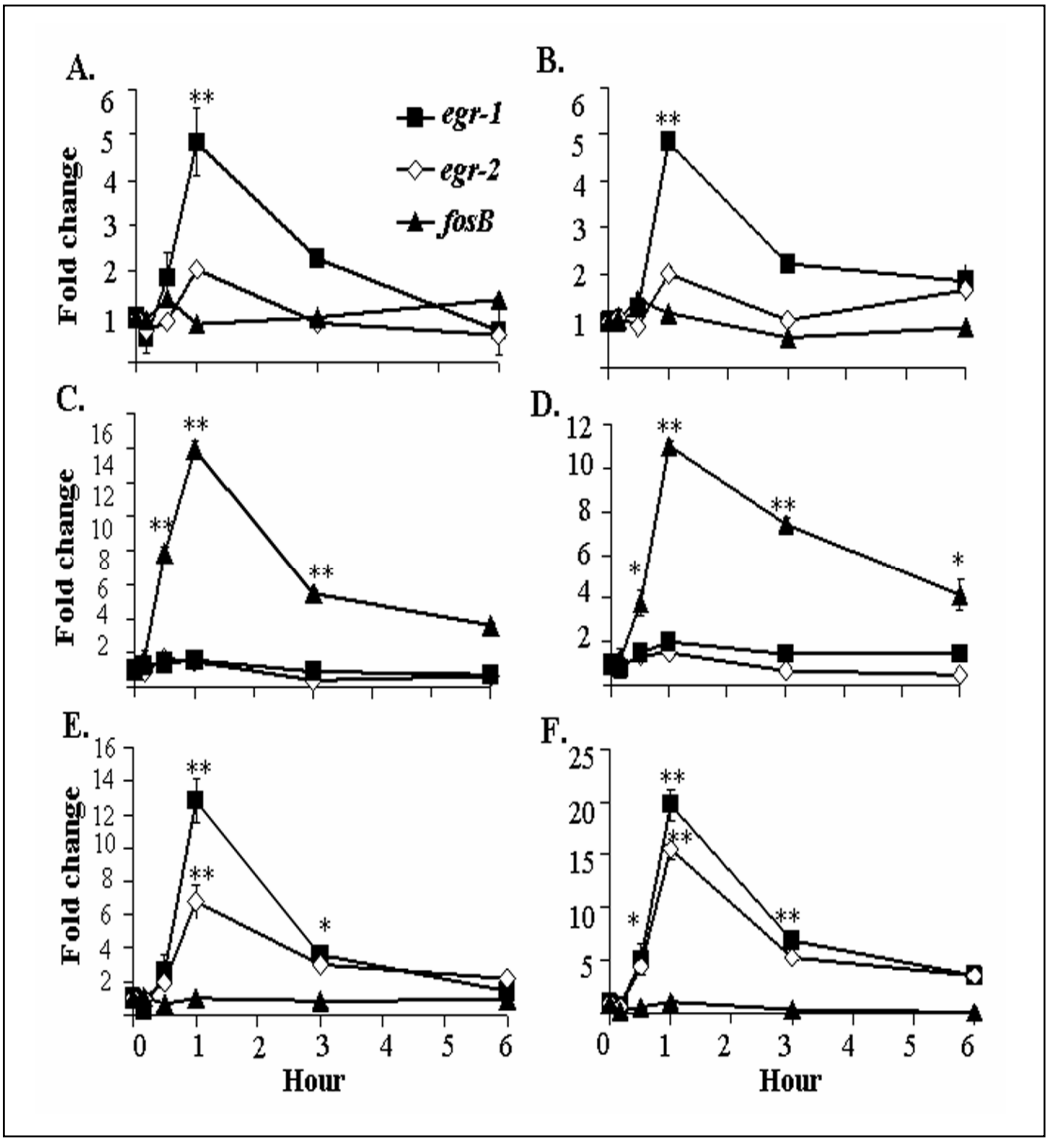

Figure 4.9. Kinetic analyses of the regulations of early response genes by GDNF and NTN in GFRα2 isoform transfectants. Fold change of mRNA expressions of early response genes in cells expressing GFRα2a (**A**), GFRα2b (**C**) and GFRα2c (**E**) when stimulated with GDNF, and GFRα2a (**B**), GFRα2b (**D**) and GFRα2c (**F**) when stimulated with NTN at the designated period of time. The expression levels were measured by quantitative real time PCR. Similar results were obtained for more than three separate experiments. Error bars indicate standard deviations of triplicate measurements from one study. Significant differences in expression of genes between ligand stimulated and control samples were calculated using paired Student's t-test. A value of $P<0.05$ was considered significant (**$P<0.001$, *$P<0.05$).

4.2.6 Neurite outgrowths were induced by GFRα2a and GFRα2c, but not GFRα2b

Neuro2A is an excellent *in vitro* model system for studying signaling pathways mediating neurite outgrowth. Under normal growth conditions, most Neuro2a cells spontaneously sprout a basal level of neurites. However, treatments with a variety of stimuli cause these cells to develop extensive neurites similar to changes observed in primary neuronal cultures (Chapter 4, Background and objectives). To investigate possible morphological changes induced by the activation of the GFRα2 isoforms, the transfectants were stimulated with either GDNF or NTN.

Both GFRα2a and GFRα2c transfectants showed extensive neurite outgrowths when stimulated with either GDNF or NTN, comparable to the effects of retinoic acid (Fig. 4.10A, B). Unexpectedly, neither of the ligands induced neurite outgrowth in cells expressing GFRα2b (Fig. 4.10A, B). Immunocytochemical staining for beta III-tubulin further confirmed these observations (appendix II). Cells expressing GFRα2b extended neurite-like structures when treated with retinoic acid, indicating the potential for neurite outgrowth (Fig. 4.10). GDNF and NTN have no neuritogenic effect on control vector transfected Neuro2A cells (Fig. 4.10).

To further examine the morphological changes in these cells, two major cytoskeletal components, F-actin and high-molecular-weight neurofilament protein (NF-H), which are involved in neurite outgrowth dynamics, were visualized by fluorescent staining. With ligand (GDNF or NTN) stimulated GFRα2a and GFRα2c transfectants, NF-H-positive filopodia (axon-like processes) were relatively long and form thick threads. Protrusions with F-actin staining were observed at the edges of the thick NF-H positive axon-like elements and cell bodies (Fig. 4.11).

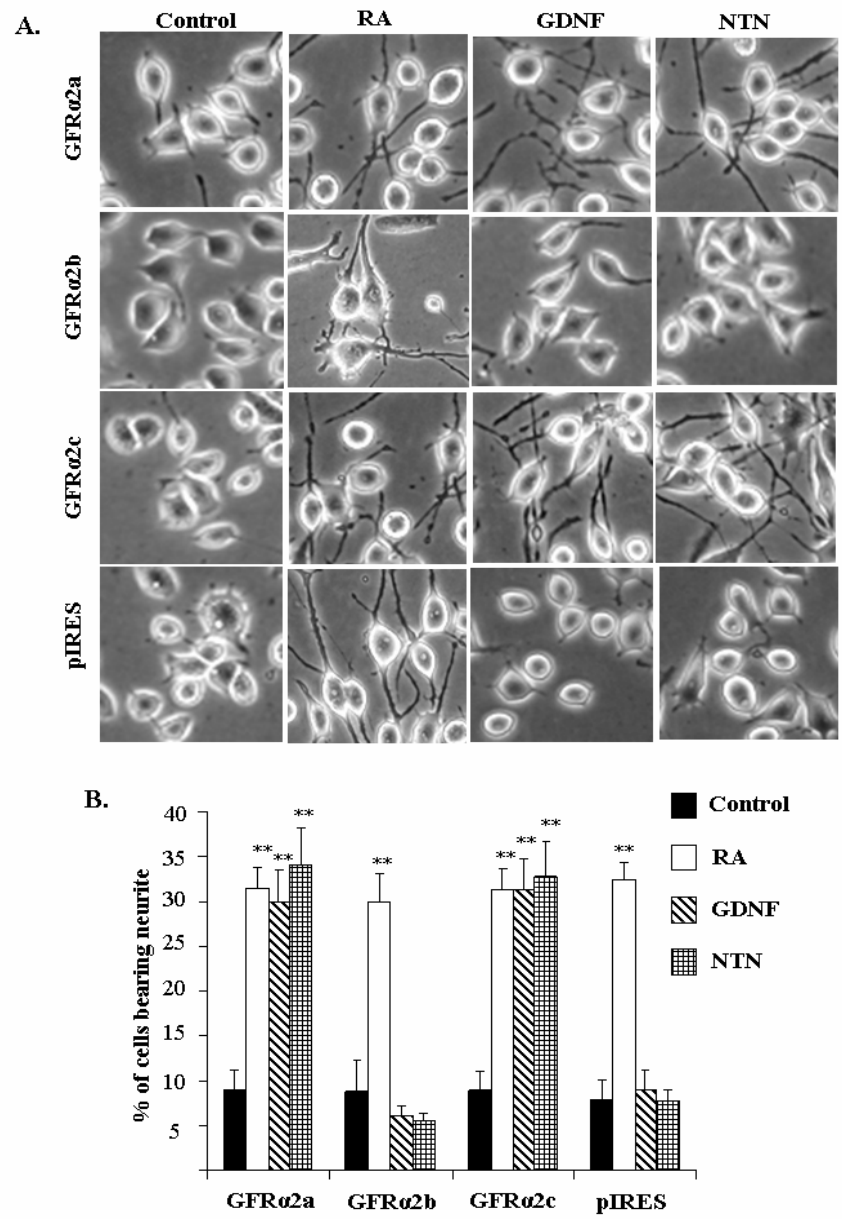

Figure 4.10. Differential neuritogenic activities of ligand activated GFRα2 isoforms. Cells were seeded on six well plates and incubated for 16-18 hours in media containing 10% serum. The cells were then exposed to GDNF or NTN (50 ng/ml) for three more days in 0.5% serum containing media. Retinoic acid (RA) (5 μM) was used as a positive control for cell differentiation. **A,** Digital phase contrasts images (magnification x200) of Neuro2A cells stably expressing GFRα2a, GFRα2b, GFRα2c or pIRES vector control when treated with RA, GDNF or NTN. **B,** Percentages of cells bearing neurites which were at least twice the length of the cells bodies. More than 600 cells were counted per well, on at least three different fields. Experiments were repeated twice with three individual clones, with similar results. Significant differences in the percentage of cells bearing neurites between ligands stimulated and control samples were calculated using paired Student's t-test (**P<0.002).

Engorgements were seen at some terminal structures which were both NF-H and F-actin positive (Fig. 4.11). Long extensions were not obvious with cells expressing GFRα2b when stimulated with either ligand. Instead, F-actin positive staining was found at the periphery of these cells where NF-H was not found to co-localize extensively (Fig 4.11f). These observations provide further evidence of the lack of neurite outgrowths in ligands stimulated cells expressing GFRα2b and the neuritogenic activities of the other two isoforms.

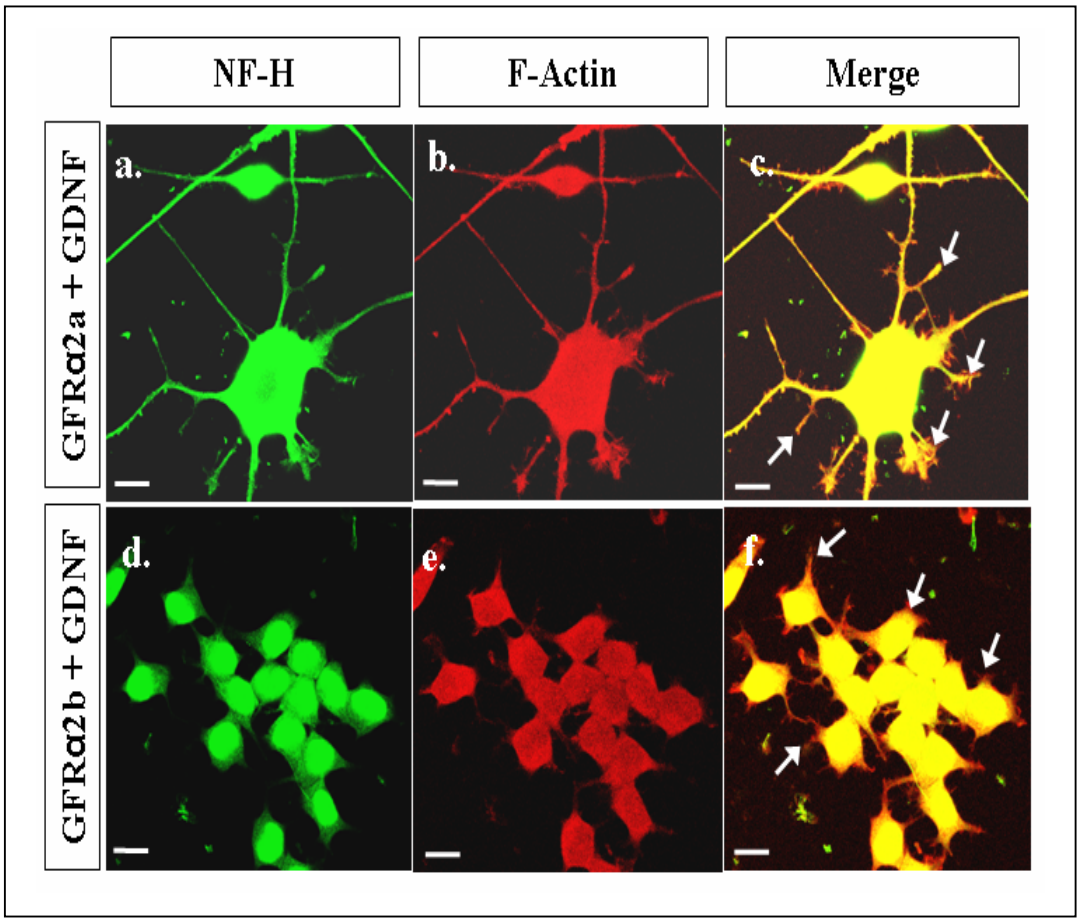

Figure 4.11. Immunocytochemistry of cytoskeletal component in ligand treated Neuro2A cells expressing GFRα2 isoforms. Confocal images for double immunostaining of heavy chain neurofilament (NH-F) (green) and F-Actin (red) in GFRα2a (a-c) or GFRα2b (d-f) transfectants treated with GDNF. Double immunostaining reveals the co-localization (yellow) of cytoskeletal components, NF-H and F-Actin along the neurites, while only F-Actin stained at growth cones (arrows). Scale bars, 20 μm.

4.3 Discussion

This study demonstrates the distinct biochemical and neuritogenic activities of alternatively spliced GFRα2 isoforms. All three GFRα2 isoforms were detected in the human cortex. Using transfected Neuro2A cells model, it has been shown that GDNF and NTN mediated differential signaling mechanism and regulated distinct early response genes in GFRα2 isoforms. Ligand activated GFRα2a and GFRα2c, but not GFRα2b, induced neurite outgrowth in Neuro2A transfectants.

The expression of GFRα2 mRNA has been reported (Golden *et al.*, 1998; Golden *et al.*, 1999; Sanicola *et al.*, 1997; Trupp *et al.*, 1998; Widenfalk *et al.*, 1997) in the cortex of the human, mouse and rat brain. However, the probes used in these studies cannot distinguish the expressions of the isoforms. In the present study, all three isoforms in the human brain regions were specifically amplified using exon overlapping primers (Too, 2003). In the human brain, all three GFRα2 isoforms were expressed at comparable levels, with GFRα2c significantly higher than the other two isoforms. Compared to the other regions of the human brain, the cortex expressed the highest level of the isoforms. The functional significance of these isoforms in the cortex is yet to be defined. Interestingly, the high expressions of the GFRα2 isoforms in the cortex, a region of the brain involved in learning complex tasks, and the observation that mice knocked out by GFRα2 show significant impairment in several memory tasks (Voikar *et al.*, 2004) may suggest a possible role of GFRα2 signaling in the development and/or maintenance of cognitive abilities that help in solving complex learning tasks.

GDNF and NTN are known to similarly activate a number of signaling pathways, including the extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, p38 mitogen-activated protein kinase (MAPK) and c-Jun N-

terminal kinase (JNK) (Ichihara *et al.*, 2004; Pezeshki *et al.*, 2003; Takahashi, 2001; Trupp *et al.*, 1999), and regulate the expressions of various immediate early response genes (Fukuda *et al.*, 2003; Pezeshki *et al.*, 2003). In this study, it is intriguing to note that the activation of specific signaling pathways, but not the early response genes, was dependent on the ligands used. For instance, GDNF was found to potently activate ERK1/2 through GFRα2a and GFRα2c in a dose and time dependent manner, but does not activate GFRα2b significantly. This was not due to the failure of GDNF to interact with GFRα2b as GDNF displaced bound ^{125}I-GDNF equally well with all three isoform transfectants. Similarly, GDNF activated AKT through GFRα2b and GFRα2c, but not through GFRα2a. However, NTN showed similar activations of ERK1/2 and AKT through all the three isoforms. Such ligands specificity in activation of GFRα2 receptor isoforms was consistent to those observed in Chapter 3.

Unlike GFRα2a and GFRα2c, GFRα2b transfectants did not induce neurite outgrowth when activated by either GDNF or NTN. Both GFRα2a and GFRα2c (but not GFRα2b) activated the early response gene, *egr-1* (also known as NGFI-A, krox-24, zif-268, and TIS-8), consistent with a role of *egr-1* in neuronal differentiation (Pignatelli et al., 1999; Knapska and Kaczmarek, 2004).

Together, the results from this study showed that the three GFRα2 isoforms have distinct biochemical and neuritogenic activities, at least in Neuro2A cells.

Chapter 5 Part III: Ligand induced, RhoA dependent inhibitory activities of GFRα2b isoform

5.1 Background and objectives

In the previous chapter, we have shown the differential biochemical and neuritogenic activities of GFRα2 isoforms when activated by the ligands, GDNF or NTN. Besides the distinct regulations of MAPK, AKT signaling and early response genes, there were also startling phenotypic differences with the activation of the GFRα2 isoforms. Both GDNF and NTN have been shown to induce neurite outgrowth in some neuronal cells (Baloh *et al.*, 2000; Lin *et al.*, 1993; Wanigasekara and Keast, 2005; Zihlmann *et al.*, 2005). Despite the presence of functional cognate receptors, both GDNF and NTN failed to induce neurite outgrowths in other neuronal cells (Hansford and Marshall, 2005; Kobori *et al.*, 2004). In view of the results where ligand activation of GFRα2a and GFRα2c, but not GFRα2b, induced neurite outgrowth in Neuro2A cells (Chapter 4), it is possible that the preferential expression of these spliced isoforms in some of these neuronal cells may underlie the phenotypic differences observed with ligand stimulation.

The Rho family of small GTPases is intimately involved in various processes involving actin cytoskeleton reorganization in eukaryotic cells, including neurite outgrowth and differentiation (Govek *et al.*, 2005). There are at least 20 Rho GTPases encoded in the human genome (Wherlock and Mellor, 2002). RhoA (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42) are the three well-studied members of the Rho family. Recent findings show that Rho GTPases play important roles in the regulation of neuronal morphogenesis, including axon outgrowth and guidance, polarity, dendrite elaboration and synapse plasticity and formation (Luo, 2000). While Rac1 and Cdc42 have been shown to positively regulate neuritogenic activities, RhoA has been shown to be a negative regulator. Attractive cues regulate Rac1 and Cdc42 activities, while

repulsive cues activate RhoA activity, which in turn transduce intracellular signals and differentially regulate growth cones actin dynamics (Dickson, 2001). RhoA and its effector ROCK are the important mediator of neurite growth inhibition regulated by various inhibitory factors, including Nogo, myelin protein MAG, and lysophosphatidic acid (LPA). Although the downstream signaling/s that link/s RhoA and ROCK to the actin cytalskeletal reorganization remain to be elucidated, LIM kinase (LIMK) appears to be involved. LPA activations of RhoA and ROCK have been shown to phosphorylate and activate LIMK, which in turn phosphorylate and subsequently inactivate the actin-associated protein, cofilin (Maekawa *et al.*, 1999; Ohashi *et al.*, 2000). Cofilin is the key regulator of actin dynamics at the leading edge of neurite growth cone. It is a small (19kDa) ubiquitous protein, that binds and dissociates actin monomer from the pointed end of actin filaments, thus generating new actin barbed ends for polymerizations of actin filaments (DesMarais *et al.*, 2005). Such regulated polymerizations and depolymerizations of actin filaments are important for neurite growth cones dynamics. Phosphorylation and inactivation of cofilin will cause rearrangement of actin cytoskeletal, leading to growth cone collapse and outgrowth inhibition (Hsieh *et al.*, 2006).

Eight Rho proteins (Rac1, Rac2, RhoA, RhoB, RhoG, TC10, TCL, and Cdc42) are known to be activated by RTKs, including RET (Schiller, 2006). In response to GDNF, several proteins in neuroblastoma cells are phosphorylated on tyrosine and reorganized actin cytoskeleton which are mediated by both Rho-dependent and -independent signaling pathways (Murakami *et al.*, 1999). However, it is not known if this response to GDNF is mediated by the activation of specific GFRα isoforms.

This chapter focuses on the mechanisms underlying the functions of GFRα2b isoform and neuritogenic activity. Using co-expression models, ligand activated

GFRα2b was found to inhibit neurite outgrowth induced by other GFRα receptor isoforms. Furthermore, ligand activated GFRα2b also attenuated retinoic acid induced neurite outgrowth. These inhibitory effects were found to be RhoA dependent.

5.2 Results

5.2.1 GFRα2b inhibited neurite outgrowths mediated by other GFRα2 isoforms

As GFRα2b transfectant did not induce neurite outgrowth when stimulated with ligand, the possibility that this isoform may affect the morphological changes induced by the activation of GFRα2a and GFRα2c was explored. Stably transfected Neuro2A cells co-expressing GFRα2b and GFRα2a or GFRα2c were established. Using the pIRES-bicistronic vector, GFRα2a or GFRα2c was expressed in the 5' multiple cloning site (MCS-A), while GFRα2b was expressed in the 3' multiple cloning site (MCS-B). For control, cells with only GFRα2a or GFRα2c expressed using pIRES-bicistronic vector, at MCS-A, showed similar neurite outgrowths activities as previously obtained (data not shown). This was also the case for GFRα2b expressed in MCS-B (data not shown).

Ligand induced stimulation of cells co-expressing GFRα2a and GFRα2b showed significantly less neurite outgrowth (Fig 5.1). However, these cells extended neurite when treated with retinoic acid. Similarly, ligand stimulation of cells co-expressing GFRα2b and GFRα2c showed significantly fewer neurite outgrowths (Fig 5.1). These observations suggest that GFRα2b, when co-expressed, antagonizes ligand induced neurite outgrowths of other GFRα2 isoforms.

5.2.2 GFRα2b inhibited neurite outgrowth mediated by GFRα1a

Extending this finding, the possible inhibitory effect of the activation of GFRα2b on the neurite outgrowth induced by ligand in cells co-expressing GFRα1a was explored. Cells expressing only GFRα1a showed significant neurite outgrowths when stimulated by GDNF, NTN or retinoic acid (Fig 5.2). Interestingly, when stimulated

by either GDNF or NTN, cells co-expressing GFRα2b and GFRα1a showed significantly less neurite outgrowths. These observations indicate that the activation of GFRα2b inhibits neurite outgrowths induced by the activation of GFRα2a, GFRα2c, and even the structurally related GFRα1a.

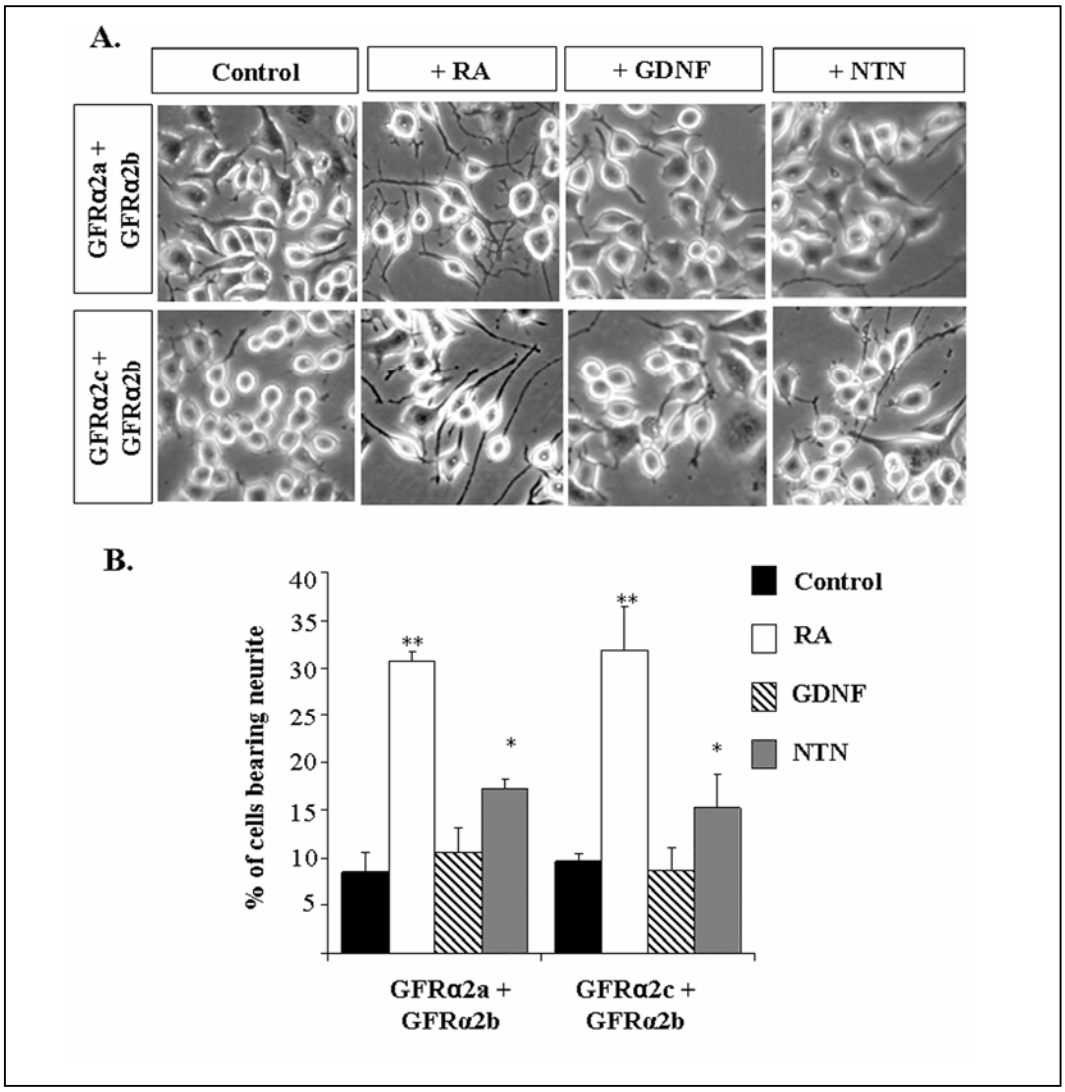

Figure 5.1. GFRα2b inhibited neurite outgrowths of GFRα2a and GFRα2c in co-expression models. To investigate effect of GFRα2b on ligand-induced neurite outgrowths of other GFRα2 isofroms, GFRα2b was stably co-expressed with GFRα2a or GFRα2c in Neuro2A cells. **A,** Phase contrasted images (magnification x200) of cells treated with retinoic acid (RA) (5 μM), GDNF or NTN (50 ng/ml). **B,** Percentages of cells bearing neurite were counted for cells bearing neurite twice longer than cells body. Experiments were repeated twice in three separate clones, with parallel results. Significant differences in percentage of cells bearing neurites between ligand stimulated and control samples were calculated using paired Students t-test (**P<0.002, *P=0.05).

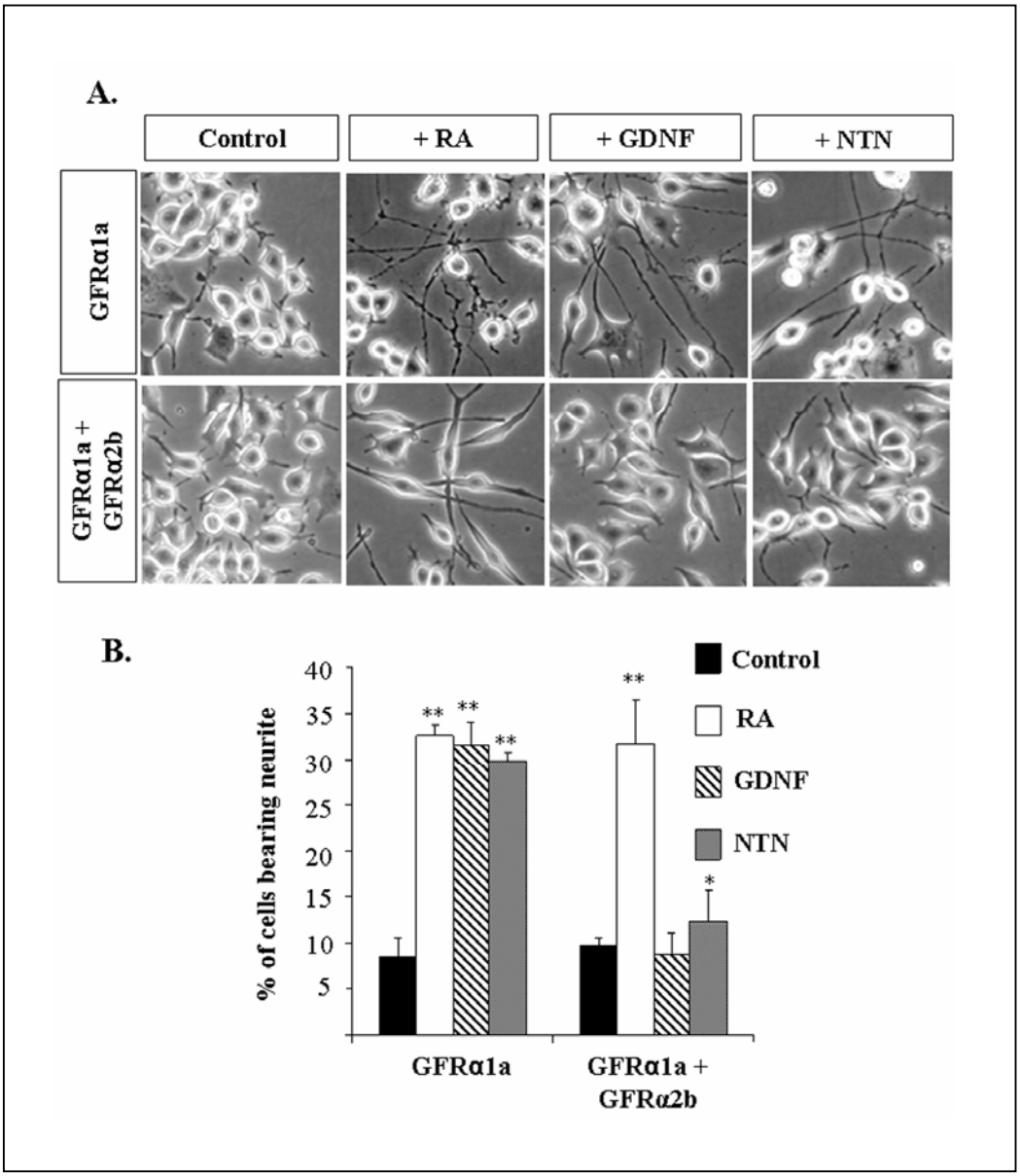

Figure 5.2. Ligand activated GFRα2b antagonized neurite outgrowths induced by ligand activated GFRα1a in co-expression model. A, B, Antagonizing effects of GFRα2b on ligand activation of GFRα1a isoforms mediated neurites outgrowths. Neuro2a cells were stably expressing GFRα1a or co-expressing GFRα1a with GFRα2b (GFRα1a+ GFRα2b). **A,** Phase contrast images (magnification x200) of cells treated three days with retinoic acid (RA) (5 μM), GDNF or NTN (50 ng/ml). **B,** Percentages of cells bearing neurite were counted for cells bearing neurite twice longer than cells body. Experiments were repeated twice in three separate clones, with parallel results. Significant differences in percentage of cells bearing neurites between ligand stimulated and control samples were calculated using paired Students t-test (**P<0.002).

5.2.3 Knock-down of GFRα2b resulted in an increase in neurite outgrowths

The above observations of the GFRα2b induced inhibition of neurite outgrowth were extended by investigating the BE(2)-C cells that have previously been shown to endogenously express GFRα2 receptors (Chapter 4) (Kobori *et al.*, 2004; Yoong *et al.*, 2006). These cells co-express GFRα2 isoforms, with comparable levels of GFRα2a and GFRα2b (Fig. 5.3A) and significantly lower level of GFRα2c (data not shown). It has previously been shown that retinoic acid, but not GDNF or NTN induce neurite outgrowth in BE(2)-C cells (Chapter 3) (Kobori *et al.*, 2004; Yoong *et al.*, 2006). From the above evidence of GFRα2b inhibiting the neuritogenic activity of GFRα2a in the co-expression model, it is reasonable that a similar mechanism may underlie the effects of GDNF or NTN in BE(2)-C cells.

In order to test the hypothesis that the activation of GFRα2b may inhibit neurite outgrowths induced by GFRα2a or GFRα2c in BE(2)-C cells, the expression of GFRα2b was silenced using siRNA. As GFRα2b has no unique sequences when compared to GFRα2a, the design of a GFRα2b isoform specific siRNA posed a significant challenge. A series of siRNA duplexes were then designed with sequences overlapping exons 1 and 3 of GFRα2b (refer to Table 9.2, Chapter 9, Materials and Methods). Of the five designs tested, only the siGFRα2b-13+7 showed significant discrimination in silencing GFRα2b over GFRα2a (Fig. 5.3B). This particular siRNA design, siGFRα2b-13+7, down regulated the expression of GFRα2b to less than 10% of the control, with no significant reduction in the expression of GFRα2a.

When GFRα2b expression was silenced, the BE(2)-C cells extended neurite-like structures when stimulated with either GDNF or NTN (Fig. 5.3C, D). This

observation supports the notion that the activation of GFRα2b inhibits neurite outgrowth induced by ligands stimulation of GFRα2a.

5.2.4 Signaling and biochemical activities of GFRα2 isoforms in the co-expression model

To further investigate the signaling and biochemical events underlying ligand activation of GFRα2b in the co-expression model, the stimulation of MAPK (ERK1/2) was first examined. Previous data showed that GDNF stimulated ERK1/2 phosphorylation in GFRα2a or GFRα2c, but not GFRα2b transfectants (Chapter 4, Fig. 4.5, 4.6). In the co-expression models, both GDNF and NTN induced rapid and transient phosphorylations of ERK1/2 (Fig. 5.4A).

Interestingly, when stimulated with either GDNF or NTN, there were no changes in the expression of either *egr-1* or *egr-2*. However, significant up-regulations of the expression of *fosB* were observed in the co-expression of GFRα2b with GFRα2a (Fig. 5.4B, C) or with GFRα2c (Fig. 5.4D,E). These observations showed that the activation of co-expressed GFRα2b with either GFRα2a or GFRα2c resulted in the activation of *fosB*, an early response gene, reminiscent of that observed in GFRα2b transfected alone (Fig. 4.9).

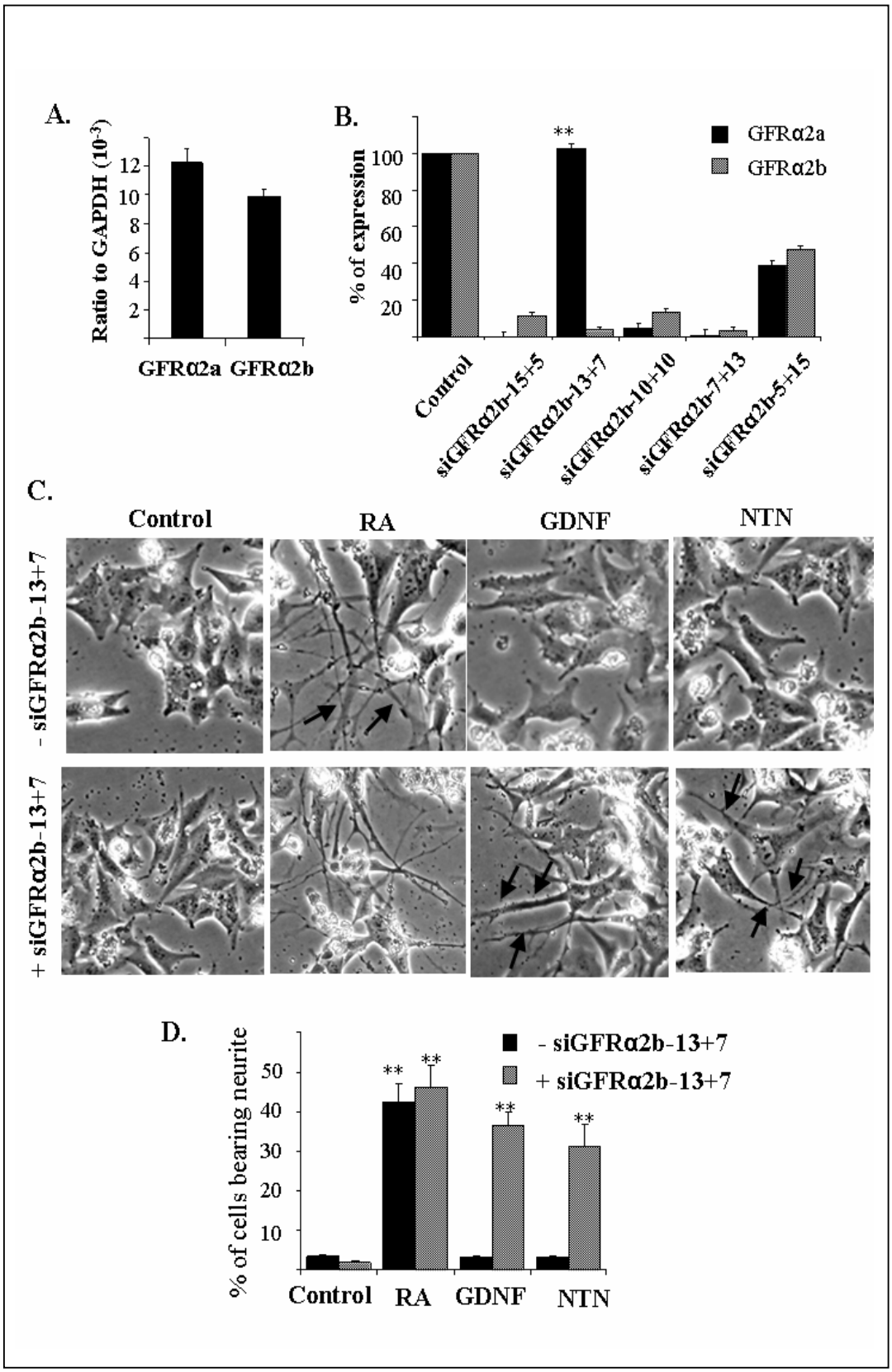
A.
Ratio to GAPDH (10^{-3})
12
10
8
6
4
2
GFRα2a GFRα2b
B.
% of expression
100
80
60
40
20
**
GFRα2a
GFRα2b
Control
siGFRα2b-15+5
siGFRα2b-13+7
siGFRα2b-10+10
siGFRα2b-7+13
siGFRα2b-5+15
C.
Control
RA
GDNF
NTN
- siGFRα2b-13+7
+ siGFRα2b-13+7
D.
% of cells bearing neurite
50
40
30
20
10
**
- siGFRα2b-13+7
+ siGFRα2b-13+7
Control
RA
GDNF
NTN

Figure 5.3. Silencing of GFRα2b expression in human BE(2)-C cells. A, The expression levels of GFRα2a and GFRα2b in BE(2)-C cells were determined using quantitative real time PCR. **B,** Effects of various designs of siRNA sequences on the expressions of GFRα2a and GFRα2b in BE(2)-C. siRNA duplex (20 pmol) was transfected into cells and total RNA was harvested six hours later. The expressions of GFRα2a and GFRα2b were then measured by quantitative real time PCR. Significant differences between the expression of the two isoforms after silencing with each of the siRNA designs were calculated using paired Students t-test (**P=0.001). **C, D,** Neurite outgrowths of BE(2)-C cells after silencing of GFRα2b. **C,** Cells were stimulated with retinoic acid (RA) (5 μM), GDNF or NTN (50 ng/ml) in the absence or presence of siGFRα2b-13+7. Pretreatment of cells with siGFRα2b-13+7 and subsequent stimulation with GDNF or NTN resulted in the formation of neurite-like structures (arrows). (Phase contrast image, magnification x200) **D,** Percentages of cells bearing neurites which were at least twice the length of the cells bodies, with or without presence of siRNA, siGFRα2b-13+7. Similar results were obtained from replicates of three individual experiments. Significant differences in percentage of cells bearing neurites between ligand stimulated and control samples were calculated using paired Students t-test (**P<0.002, *P<0.05)

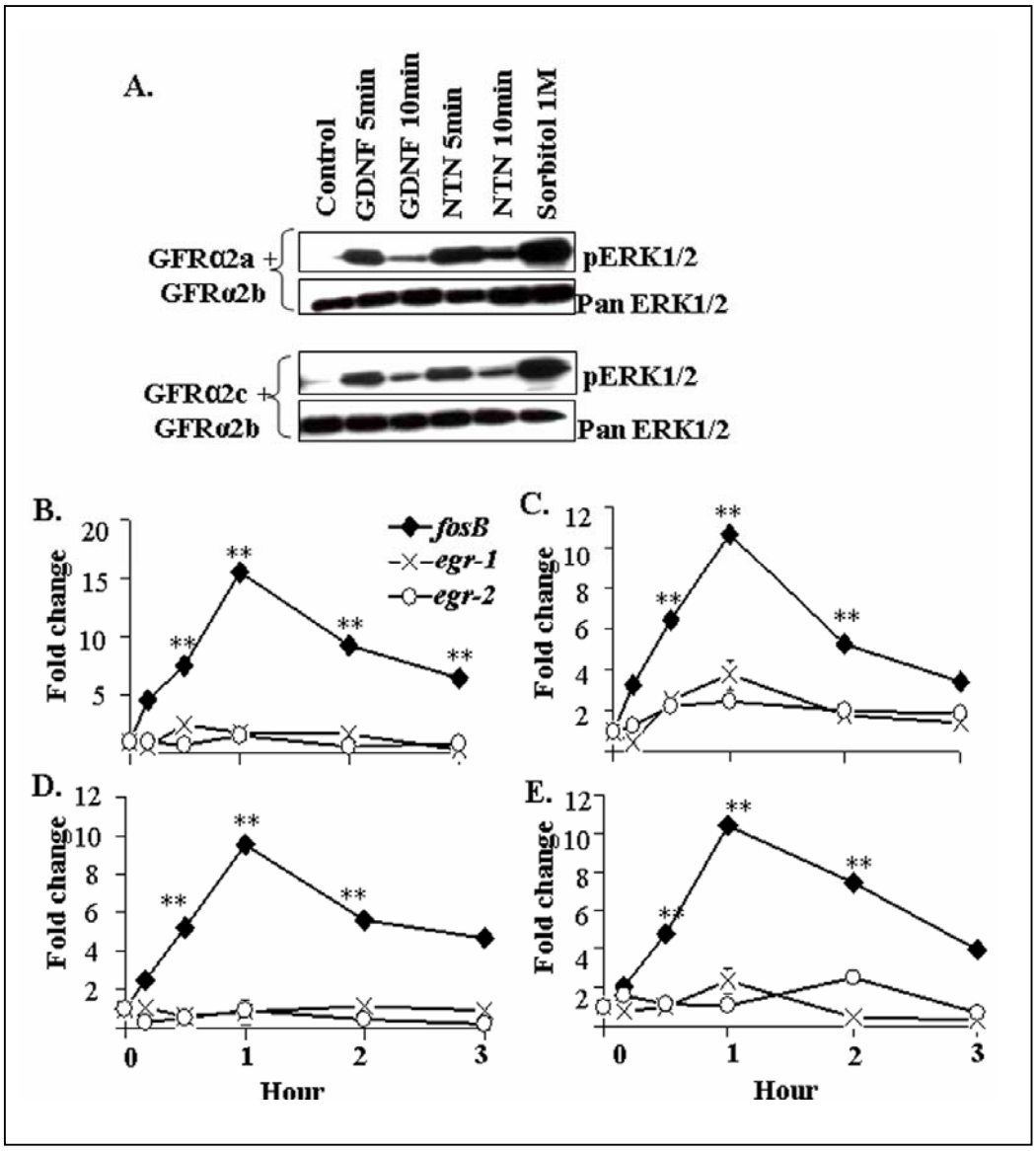

Figure 5.4. ERK1/2 signaling and the regulation of early response genes in the Neuro2A cells co-expressing GFRα2b with either GFRα2a or GFRα2c. A, Western blot analyses of the activation of ERK1/2. Neuro2A cells stably co-expressing the isoforms, GFRα2a and GFRα2b (GFRα2a + GFRα2b), or GFRα2c and GFRα2b (GFRα2c + GFRα2b) were treated with GDNF, NTN or Sorbitol for the period of time indicated. Phospho-specific antibodies to ERK1/2 were used for detection and the blots reprobed with pan antibody serving as controls for protein loadings. **B-E,** Kinetic analyses of GDNF or NTN regulated expressions of early response genes in the co-expression models. Expressions of *fosB*, *egr-1*, and *egr-2* were measured with quantitative real time PCR in cells stably co-expressing GFRα2a with GFRα2b when stimulated with GDNF (**B**) or NTN (**C**); Cells stably co-expressing GFRα2c with GFRα2b were stimulated with GDNF (**D**) or NTN (**E**). Significant differences in expression of genes between ligand stimulated and control samples were calculated using paired Student's t-test. A value of $P<0.05$ was considered significant (**$P<0.001$, *$P<0.05$).

5.2.5 GFRα2b inhibited retinoic acid induced neurite outgrowth

Next, we examined the possibility that GFRα2b may affect neurite outgrowth induced by retinoic acid, a non-GFLs stimulus. Using Neuro2A expressing GFRα2b, retinoic acid treatment resulted in extensive neurite outgrowths. Both GDNF and NTN dramatically reduced the number of cells bearing neurite-like structures (Fig. 5.5). These suggest that GFRα2b may have a broad inhibitory activity on neurite outgrowth stimulated by different agents.

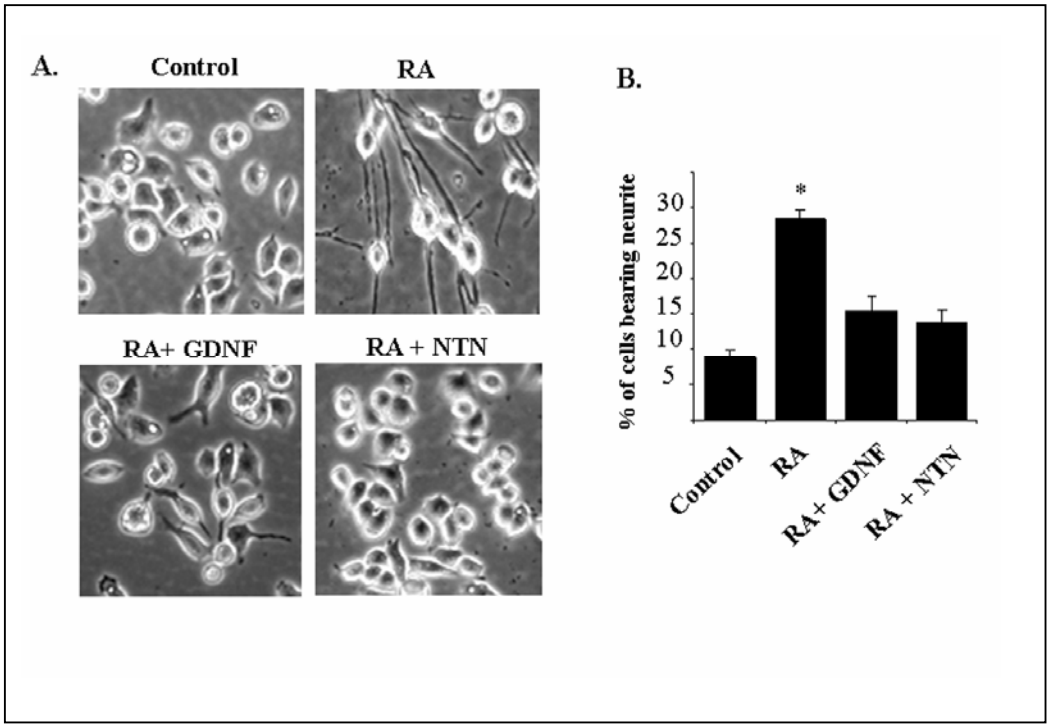

Figure 5.5. Ligand activated GFRα2b antagonized neurite outgrowth induced by retinoic acid. A, B, Retinoic acid (RA) (5 μM) induced neurite outgrowth in GFRα2b expressing Neuro2A cells. When treated together with GDNF or NTN (50 ng/ml), neurite outgrowth induced by RA is significantly attenuated. **A,** Phase contrast images (magnification x200) of Neuro2A cells stably expressing GFRα2b, treated with RA, GDNF or NTN for three days. **B,** Percentages of cells bearing neurite were counted for cells bearing neurite twice longer than cells body. Experiments were repeated twice in three individual clones, with similar results. Significant differences in percentage of cells bearing neurites between ligand stimulated and control samples were calculated using paired Students t-test (*P<0.002).

5.2.6 Ligand induced GFRα2b neurite inhibition is RhoA dependent

The Rho family of small GTPases and its associated regulators have been implicated in the modulation of neurite formation, axonal pathfinding, and dendritic arborization (Dickson, 2001; Van Aelst and Cline, 2004). Thus, it is of interest to examine the possibility that GFRα2b may activate the Rho family of GTPases. The possible involvement of Rho-associated kinase, ROCK, which is known to be an effector of RhoA in the negative regulation of neurite outgrowth, was also investigated. To address these issues, effects of Rho inhibitor, exoenzyme C3 transferase, and ROCK inhibitor, Y27632, on neurite outgrowth of GFRα2 isoforms co-expressing cells were studied. Neuro2A cells have previously been shown to respond to LPA, resulting in the inhibition of neurite outgrowths through the RhoA-ROCK dependent mechanism (Sayas *et al.*, 2002). Hence, LPA was used as a positive control for the effectiveness of Rho-ROCK inhibitors.

When stimulated with GDNF or NTN, Neuro2a co-expressing GFRα2a and GFRα2b (Fig. 5.6A) or GFRα2c and GFRα2b (Fig. 5.6B) did not extend neurite-like structures. However, a significant number of these cells extended neurite-like structures in the presence of the Rho antagonist, exoenzyme C3 transferase, suggesting the involvement of Rho family of GTPases in the inhibitory effects of GFRα2b (Fig. 5.6A, B). C3 transferase was also found to inhibit LPA effects on retinoic acid induced neurite outgrowth. At the concentration level of C3 transferase used in this study, no significant cell death was observed (data not shown).

Using the ROCK inhibitor, Y27632, the inhibitory activity of LPA on retinoic acid induced neurite outgrowth was significantly attenuated (Fig. 5.6). However, the same concentration of Y27632 (10 μM) did not attenuate the inhibitory activity of GFRα2b in co-expression with GFRα2a (Fig. 5.6A) or GFRα2c (Fig. 5.6B). Higher

concentrations of Y27632 (20 μM) resulted in significantly higher backgrounds of neurite outgrowth, thus complicating the interpretation of this study.

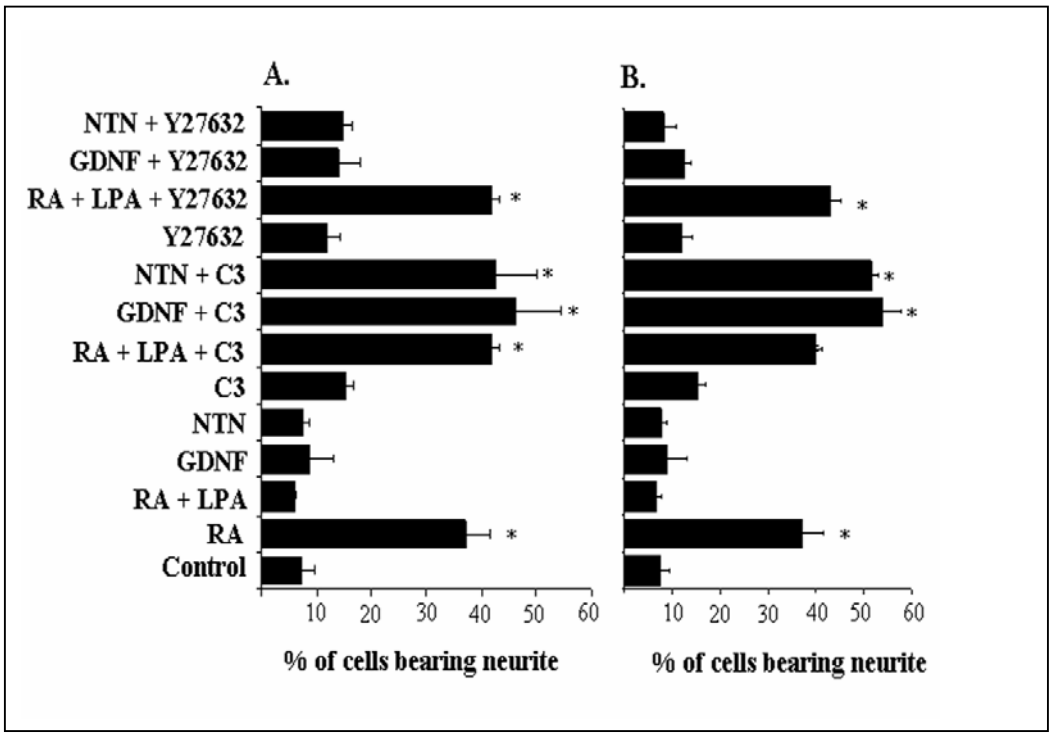

Figure 5.6. Effects of RhoA and ROCK inhibitors in ligand induced neurite outgrowth of GFRα2 isoforms co-expression models. A, B, Effects of RhoA inhibitor, exoenzyme C3 transferase (1 μg/ml) and ROCK inhibitor, Y27632 (10 μM), on ligand induced neurite outgrowth in co-expression models of GFRα2a and GFRα2b (GFRα2a+ GFRα2b) (**A**), or GFRα2c and GFRα2b (GFRα2c+ GFRα2b) (**B**). Lysophosphatidic acid (LPA) was used as a positive control in this study. LPA (10 μM) antagonized neurite outgrowth induced by 5 μM retinoic acid (RA), such neurite inhibition of LPA was attenuated by C3 (1 μg/ml) and Y27632 (10 μM). Mean ± SD was calculated from results obtained in triplicates. The effects of RhoA and ROCK inhibitors were compared to the effects of the inhibitors alone. With the concentrations of inhibitors used, no significant cell deaths were observed. Significant differences in percentage of cells bearing neurites were calculated between ligand stimulated and control samples, using paired Student's t-test (*$P \leq 0.01$).

In order to investigate the possible involvement of RhoA in the inhibitory effects of GFRα2b, an attempt was made to pull down activated RhoA from cell lysates using GST-Rhotekin and subsequently immunoblotted for RhoA. Similar to the effects of LPA, when GFRα2b was stimulated with either NTN or GDNF, it was

found to activate RhoA significantly (Fig. 5.7). Neuro2A expressing GFRα2a, GFRα2c or pIRES vector control did not activate RhoA significantly when stimulated with these ligands. This observation is consistent with the suggestion that RhoA and/or other Rho GTPases may be involved in the inhibition of neurite outgrowth mediated through GFRα2b.

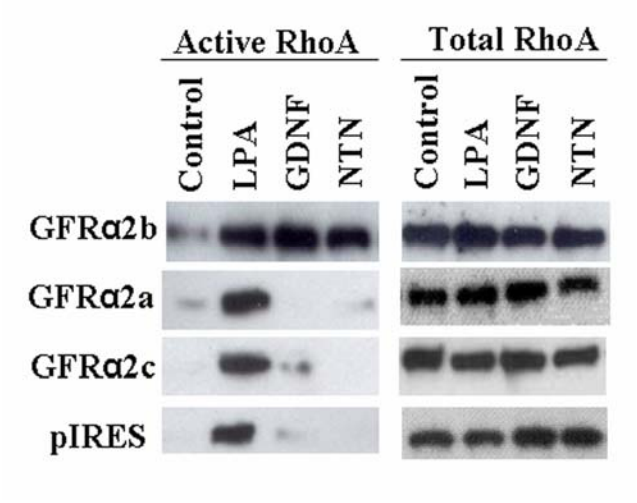

Figure 5.7. Analyses of RhoA activation in Neuro2A cells transfected with GFRα2 isoforms or pIRES control. After a 10-minute treatment of LPA (10 μM), GDNF or NTN (50 ng/ml), GTP-bound RhoA was pulled down from cells lysates using GST-Rhotekin and immunoblotted for RhoA. LPA served as a positive control for RhoA activation. Blotting of total RhoA in cells lysate showed similar loading of cell lysates for pull down assays.

The involvement of RhoA in the activation of GFRα2b was not restricted to inhibiting GFRα1a, GFRα2a or GFRα2c induced neurite outgrowths, but also to those induced by retinoic acid (Fig. 5.8A). Similar to the above observations, the inhibitory effects of GFRα2b on retinoic acid induced neurite outgrowth appear to be mediated in a Rho dependent manner (Fig 5.8A). Furthermore, the inhibition of ROCK may be sufficient to oppose the effects of LPA but not that of GFRα2b on retinoic acid induced neurite outgrowths (Fig. 5.8B).

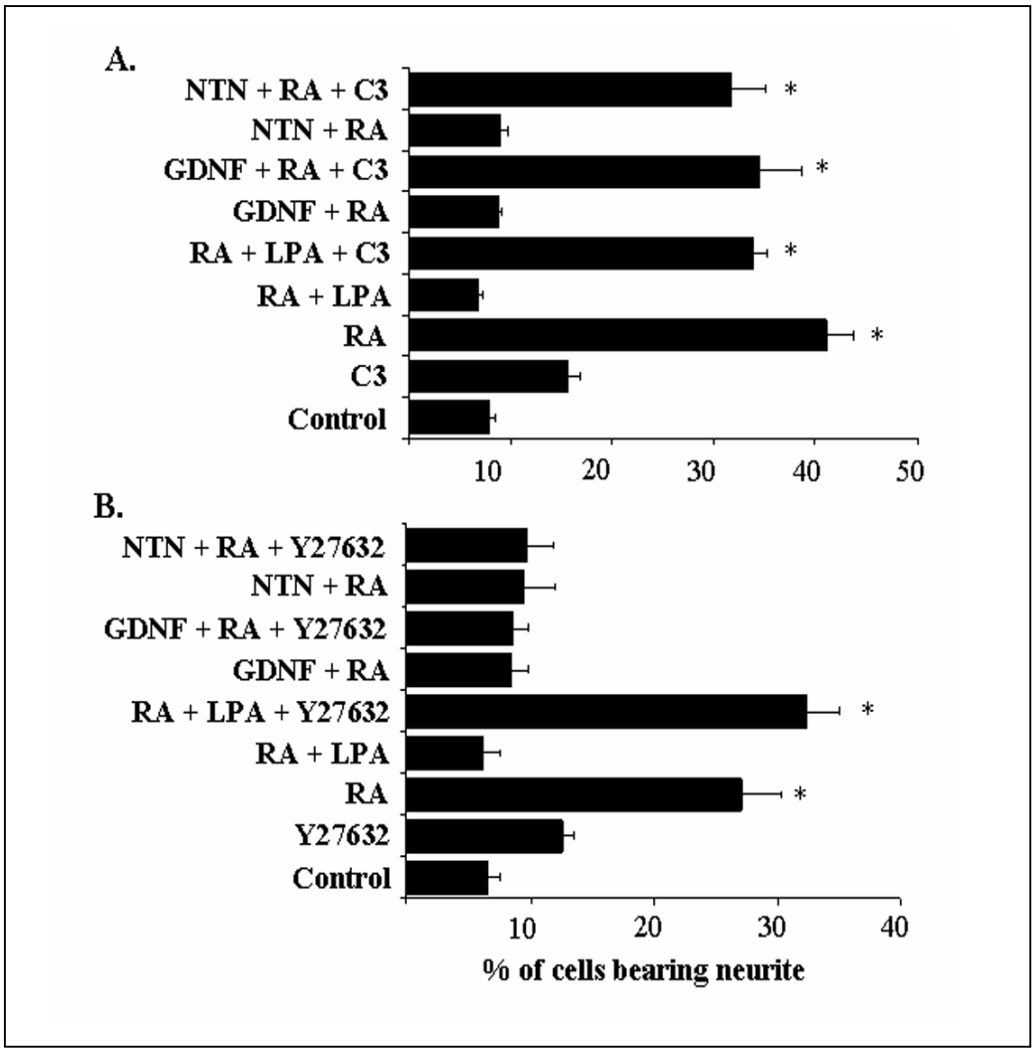

Figure 5.8. Effects of RhoA and ROCK inhibitors on GFRα2b inhibition of retinoic acid (RA) induced neurite outgrowth. A, RhoA inhibitor, exoenzyme C3 transferase (1 μg/ml) inhibited the ligand activated GFRα2b attenuation of neurite extension induced by retinoic acid (RA) (5 μM). The same concentration of exoenzyme C3 transferase also attenuated Lysophosphatidic acid (LPA) (10 μM) inhibition of RA induced neurite extension. **B,** Lack of effect of ROCK inhibitor, Y27632, on the ligand activated GFRα2b inhibition of RA induced neurite extension. The same concentration of Y27632 (10 μM) significantly attenuated the neurite outgrowth inhibition induced by LPA. Mean ± SD was calculated from results obtained in triplicates. The effects of RhoA and ROCK inhibitors were compared with the effects of the inhibitors alone. With the concentrations of inhibitors used, no significant cell deaths were observed. Significant differences in percentage of cells bearing neurites were calculated between ligand stimulated and control samples, using paired Student's t-test (*$P \leq 0.01$).

To further confirm the involvement of RhoA in GFRα2b inhibitory activities, the effect of RhoA dominant negative mutant (RhoA-DN) was investigated (Fig. 5.9). Expression of RhoA-DN in GFRα2b cells resulted in an increase in the percentage of cells bearing neurite, in ligand treated GFRα2b cells (Fig. 5.9B). It also abolished the GFRα2b inhibition on RA-induced neurite outgrowth. Neurite outgrowths were observed in cells stained positive for both HA-tagged RhoA-DN and beta III tubulin (Fig. 5.9A). These results further indicate the involvement and importance of RhoA in the inhibitory activities of GFRα2b.

Actin reorganization is commonly observed in RhoA-ROCK mediated neurite retraction and growth cone collapse. The activation of RhoA-ROCK pathway by LPA leads to phosphorylation and hence deactivation of cofilin, an adaptor protein involved in actin depolymerization (Hsieh *et al.*, 2006; Huang *et al.*, 2006; Maekawa *et al.*, 1999). Here, the possibility of cofilin deactivation by ligand induced GFRα2b was also investigated. Ligand activated GFRα2b induced rapid phosphorylation of cofilin (Fig. 5.10). Increased phosphorylation of cofilin was not observed in GFRα2a or GFRα2c (data not shown). It has also been noted that the cofilin phosphorylation induced by ligand activated GFRα2b was not as intense and sustainable as compared to that induced by LPA treatment. The differences in cofilin phosphorylation induced by ligand activated GFRα2b and LPA treatment may be indicative of differences in the underlying mechanisms of neurite outgrowth inhibition.

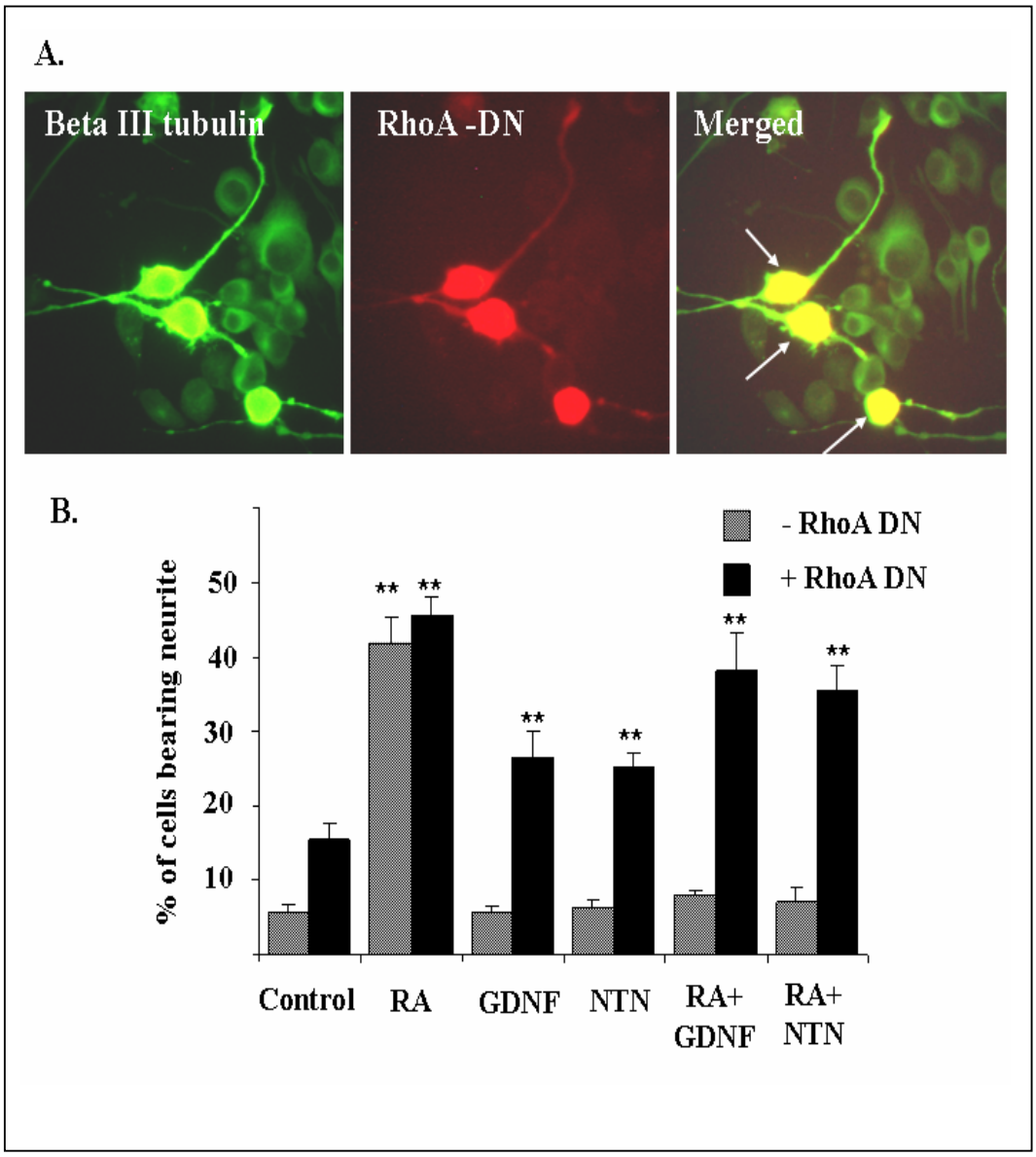

Figure 5.9 RhoA dominant negative mutant prevented inhibitory effects of GFRα2b. A, GFRα2b cells transfected with HA-tagged RhoA dominant negative mutant (RhoA DN), where differentiated with retinoic acid (RA) in presence of GDNF. Cells were stained with beta III tubulin (green) and anti-HA (red). Cells double-stained with beta III tubulin and HA-RhoA DN showed positive for neurite outgrowth (arrowhead). (maginification x200) **B,** Cells count for neurite outgrowth mediated by GFRα2b treated with or without RhoA DN. Significant differences in percentage of cells bearing neurites were calculated between ligand stimulated and control samples, using paired Students t-test (**P≤0.01).

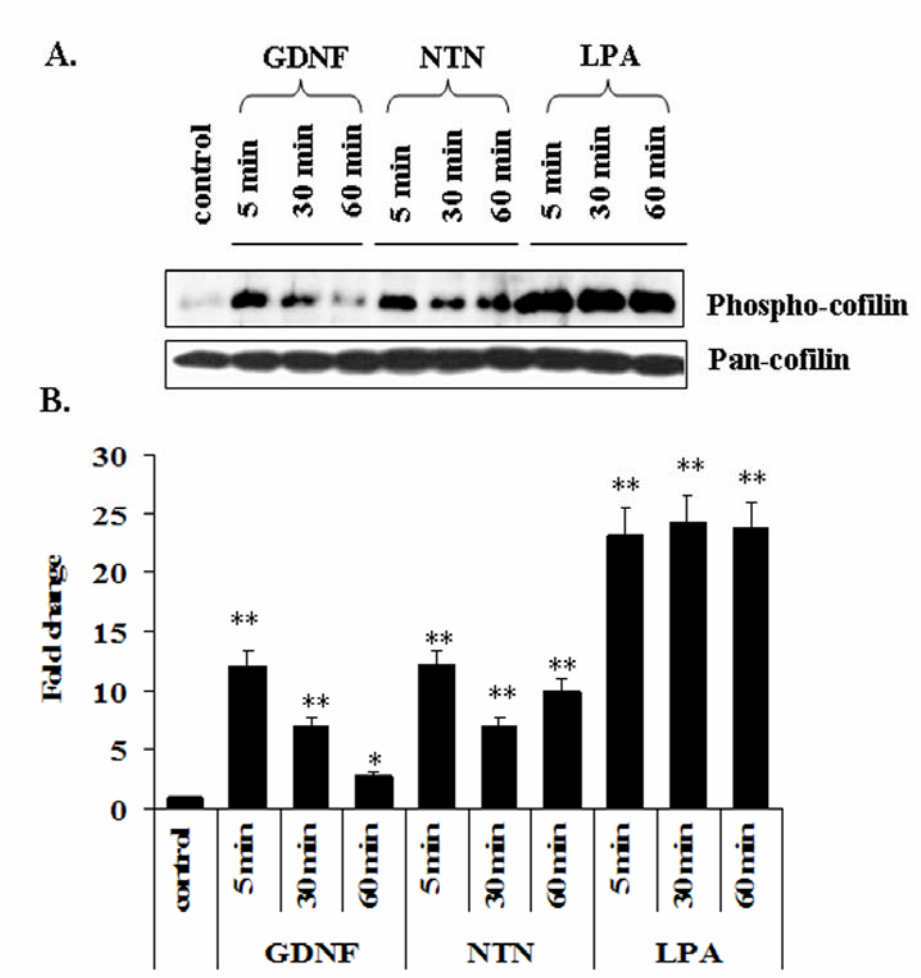

Figure 5.10. Ligand activated GFRα2b mediated phosphorylation of cofilin. Neuro2A cells stably expressing GFRα2b were treated with 50 ng/ml of GDNF or NTN, or 10 μM of Lysophosphatidic acid (LPA), for 5, 30 or 60 minutes. LPA served as a positive control for phosphorylation of cofilin. **A,** Western blots detection of phospho-cofilin in Neuro2A cells stably expressing GFRα2b were treated with GDNF, NTN or LPA. Blot was stripped and reprobed for pan-cofilin. **B,** Fold change of phosphorylated cofilin in GFRα2b cells treated with GDNF, NTN or LPA. Similar results were repeated in three independent experiments. Significant differences in fold change of phosphorylated cofilin between control and treated samples were calculated using paired Student's t-test. A value of 0.05 was considered significant (*P<0.05, **P<0.01)

5.2.7 GFRα2b may prevent but not retract neurite outgrowth

In the studies on the inhibition of retinoic acid induced neurite outgrowth by GFRα2b, GDNF or NTN was added simultaneously with retinoic acid. It would be of interest to know if inhibition of neurite outgrowth is effective if cells were first treated with retinoic acid, prior to ligand activation of GFRα2b. As was shown previously (Fig. 5.5), minimal neurite outgrowths were observed when the GFRα2b transfectants were simultaneously exposed to retinoic acid and GDNF or NTN. However, when the ligands were added one, three, or six hours after the addition of retinoic acid, significant neurite outgrowths were observed (Fig. 5.11A). This was however less as compared to cells treated with retinoic acid alone (Fig. 5.11A). These data indicate that neurite inhibition of GFRα2b may be most effective when GFRα2b is activated by ligand prior to the exposure to retinoic acid.

Besides inhibiting neurite outgrowth, LPA has also been shown to induce growth cone collapse and neurite retraction (Couvillon and Exton, 2006; Kranenburg *et al.*, 1999). This raises the interesting question of whether GFRα2b may also induce neurite retraction. Retraction assays were performed by adding LPA, GDNF or NTN to 3-day retinoic acid differentiated GFRα2b cells. Three hours later, most cells treated with LPA rounded up in shape, indicating neurite retraction (Fig. 5.11B, C). GDNF or NTN did not have such an effect on retinoic acid differentiated GFRα2b cells, suggesting the neurite did not retract (Fig. 5.11B, C).

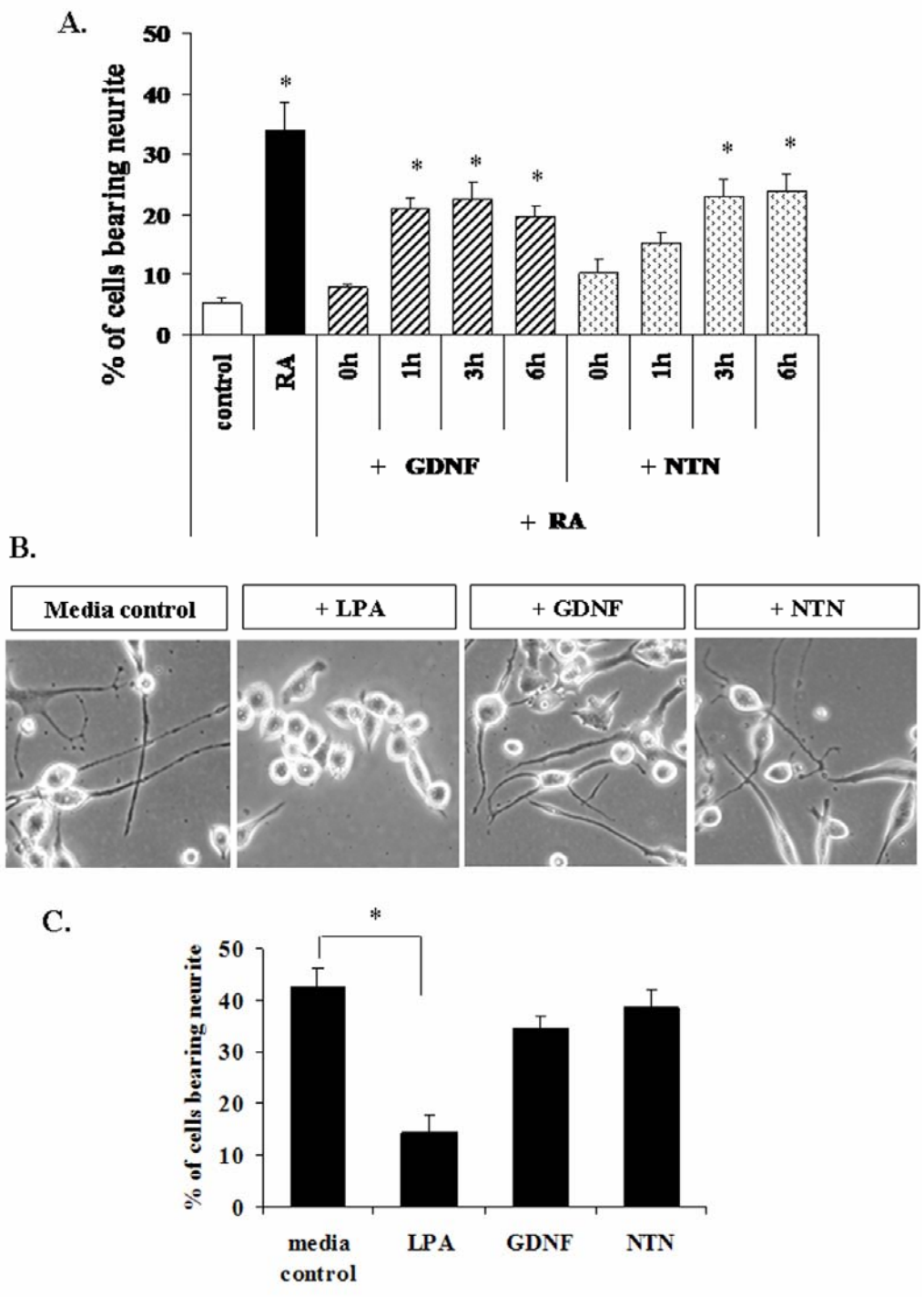
A.
% of cells bearing neurite
50
40
30
20
10
0
control
RA
0h
1h
3h
6h
0h
1h
3h
6h
+ GDNF
+ NTN
+ RA
B.
Media control
+ LPA
+ GDNF
+ NTN
C.
% of cells bearing neurite
50
40
30
20
10
0
media control
LPA
GDNF
NTN

Figure 5.11. Ligand activated GFRα2b may inhibit, but not retract neurite outgrowth mediated by Retinoid Acid. A, Effectiveness of ligand activated GFRα2b in attenuating neurite outgrowth of retinoic acid was investigated by adding GDNF or NTN (50 ng/ml), at zero hour, or one to six hours (1h, 3h, 6h) after cells treated with 5 μM of Retinoic Acid (RA). **B, C,** Studies of retraction effects of Lysophosphatidic acid (LPA) (10 μM), GDNF and NTN (50 ng/ml) in GFRα2b cells. Neuro2A cells stably expressing GFRα2b were differentiated with 5μM of Retinoic Acid (RA) for three days. By the third day, differentiation medium containing RA was removed, and replaced by media only, or media with LPA, GDNF or NTN. Cells were then incubated for three hours. Cells morphologies (magnification x200) (**B**) and percentage of cells bearing neurite (**C**) after removal of RA differentiation medium, and three-hour incubation of replacement media containing LPA, GDNF or NTN. Standard deviations are shown for triplicate wells. Significant differences in percentage of cells bearing neurites were calculated between stimulated and control samples, using paired Students t-test (*$P \leq 0.01$).

5.3 Discussion

This study reveals a novel inhibitory activity of the GFRα2b isoform, where activation of the isoform antagonized neurite outgrowths induced by GFRα1a, GFRα2a and GFRα2b receptor isoforms. Ligand activated GFRα2b isoform also attenuated neurite outgrowth mediated by retinoic acid, a non-GFL stimuli. Furthermore, the ligand induced anti-neuritogenic activities of GFRα2b were RhoA dependent.

Extending the observation where GFRα2b transfectants did not induce neurite outgrowth when activated by ligands (Chapter 4), the co-expression of GFRα2b was found to inhibit ligand induced neurite outgrowths by GFRα1a, GFRα2a and GFRα2c (Figure 5.1, 5.2). Similarly, in BE(2)-C cells endogenously expressing GFRα2b isoform, both GDNF and NTN did not significantly alter the morphology of the cells. However, the silencing of GFRα2b and subsequent treatment with either GDNF or NTN caused the cells to extend neurite-like structures (Figure 5.3). These results are consistent with GFRα2b serving a specific role in inhibiting neuritogenesis induced by some stimuli.

It is well documented that GDNF and NTN are potent trophic factors which have potent effects on neuronal differentiation, promote survival and sprouting of ventral mesencephalic dopaminergic neurons in primary cultures and other neuronal cultures (Lin et al., 1993; Akerud et al., 1999; Baloh et al., 2000; Yan et al., 2003; Wanigasekara and Keast, 2005; Zihlmann et al., 2005). The finding in this study of a particular alternatively spliced variant of GFRα2 inhibiting neurite outgrowth was unexpected.

The underlying GFRα2b inhibitory mechanism involves the Rho family of GTPases. RhoA is a member of Rho GTPases family, which includes RhoA, Rac and

Cdc42 (Luo, 2000; Van Aelst and Cline, 2004). While the involvement of Rac and Cdc42 in promoting neurite and axonal outgrowth has been shown, RhoA has been the focus in studies of molecular mechanisms for some glial-derived neurite outgrowth inhibitory factors such as Nogo-A, myelin-associated glycoprotein (MAG) (Niederost *et al.*, 2002) and lysophosphatidic acid (LPA) (Sayas *et al.*, 2002). More recent findings reveal that RhoA mediates neurite outgrowth inhibition by the reorganization of actin and microtubular network (Dickson, 2001; Leung *et al.*, 2002). Consistent with these findings is that GDNF and NTN increase the active form of RhoA in GFRα2b but not in GFRα2a or GFRα2c transfectants. The *Clostridium botulinum* C3 exoenzyme specifically ADP-ribosylates and inactivates Rho, thus increasing neurite outgrowths in GFRα2a/GFRα2b and GFRα2c/GFRα2b co-expression models. Furthermore, expression of RhoA dominant negative mutant abolished the inhibitory activity of ligand induced GFRα2b. It is interesting to note that GDNF induced RET mediated phosphorylation of focal adhesion kinase, paxillin and p130C through the activation of Rho family of GTPase and inhibited the outgrowth of neurites in TGW-I-nu cells (Murakami *et al.*, 1999). It is however, unclear if this observation was due to the activation of GFRα2b or similar isoform/s (GFRα1b, see Chapter 6).

The mechanism underlying the neurite outgrowth inhibitory activity of GFRα2b appears to differ in detail from that of LPA system. Both RhoA and ROCK are known to be involved in LPA induced inhibitory activities. Using these inhibitors, RhoA but not ROCK, was involved in the neurite inhibitory effect of GFRα2b. LPA but not the activation of GFRα2b, was capable of inducing neurite retraction in Neuro2A cells. Furthermore, the temporal profile and intensity of cofilin phosphorylation was also different between those activated by LPA, and those activated by GFRα2b. The

downstream effector(s) for RhoA underlying the mechanism of GFRα2b inhibition of neurite outgrowth remains unclear.

When compared to GFRα2a, both GFRα2b and GFRα2c show deletion of eight cysteine residues and N-glycosylation sites at the N-terminus (Wong and Too, 1998). GFR is thought to be structurally organized into three distinct domains. The N-terminal domain has previously been shown to be dispensable for ligands binding specificity and RET phosphorylation of GFRα receptors (Scott and Ibanez, 2001). Extending this observation, the N-terminal domain encoding the unique sequences of GFRα2a, GFRα2b and GFRα2c may serve to regulate distinct biochemical and cellular processes. It is tempting to speculate that the expression and interaction of specific GFRα2 receptor spliced isoforms may play an important role in neuronal differentiation involving GDNF and NTN. Recent observations where the expressions of GFRα2 isoforms are differentially regulated in Nurr1 induced dopaminergic differentiation of embryonic stem cells are consistent with this suggestion (Sonntag *et al.*, 2004).

In conclusion, the activation of RhoA and the deactivation of cofilin provide a consistent and convincing evidence for the existence of specific mechanisms underlying the ligand dependent inhibition of neurite outgrowth through GFRα2b.

Chapter 6 Part IV: Studies of inhibitory activities of GFRα1b isoform

6.1 Background and objectives

GFRα1a and GFRα2a are closely related, with 48 % amino acid identity. Both receptors share nearly complete conservation of cysteine residues, suggesting similar three-dimensional structures and possibly similar biological functions (Jing *et al.*, 1997). GFRα1 is organized into 11 exons (1234 bp) and is alternatively spliced to produce at least two isoforms, namely the GFRα1a and GFRα1b (Dey *et al.*, 1998; Shefelbine *et al.*, 1998). These 2 isoforms are highly homologous, with a difference of only 5 amino acids (140DVFQQ144) in exon 5, which is not present in GFRα1b. Both isoforms are co-expressed at significant levels in the central and peripheral nervous system and have distinct biochemical activities (Charlet-Berguerand *et al.*, 2004; Yoong *et al.*, 2005). However, it is unknown if the activation of these isoforms result in differential morphological changes.

GDNF and NTN have previously been shown to regulate neurite outgrowths in various neuronal systems where GFRα1 and GFRα2 isoforms are often co-expressed (Golden *et al.*, 1998; Golden *et al.*, 1999; Hishiki *et al.*, 1998). GFRα1a has been shown to induce neurite outgrowths in PC12 cells (Chen *et al.*, 2001; Wang *et al.*, 2004). It is unclear if specific GFRα1 isoforms contributes to morphological changes similar to that found with the GFRα2 isoforms (Chapter 5, 6).

In this section of the work, biochemical and morphological effects of ligand induced GFRα1 isoforms were investigated using Neuro2A cell model as described previously (Chapter 4, 5). Distinct neuritogenic activities between GFRα1 isoforms were observed. GFRα1a but not GFRα1b mediated ligand induced neurite outgrowth. Furthermore, GFRα1b mediated attenuation of neurite outgrowth of GFRα1a in a RhoA-ROCK dependent manner.

6.2. Results

6.2.1 Ligand activated GFRα1 isoforms mediated different early response genes

Neuro2A transfectants stably expressing GFRα1a or GFRα1b were generated similarly to that described in the GFRα2 studies (Chapter 5). In an effort to further understand the physiological functions of these isoforms, changes in the expression of the immediate early response genes (*fos* family (*c-fos, fosB*), *jun* family (*c-jun, jun-b*), *egr* family (*egr-1 to egr-4*) and GDNF inducible transcription factors *mGIF* and *mGZF1)* were investigated in response to GDNF and NTN. When stimulated with either ligand, GFRα1a and GFRα1b differentially regulated some of these early response genes over the period of 3 h of ligand stimulation (Fig. 6.1). The expressions of *egr-1*, *egr-2*, *c-fos* and *fosB,* were up-regulated when GFRα1a was stimulated with GDNF (Fig.6.1A). NTN was found to up-regulate *egr-1* expression over a period of 180 min, and transiently up-regulated the expression of *egr-2* and *fosB* (Fig. 6.1B). Except for a low but significant up-regulation of *egr-2* (2.5 fold), no obvious changes in the expression of the immediate early response genes were observed when GFRα1b was stimulated by GDNF (Fig. 6.1C). NTN was found to significantly up-regulate e*gr-2* gene expression over a period of 180 minutes, and transiently up-regulated *egr-1* (Fig. 6.1D). These results showed that the activation of GFRα1 isoforms results in the distinct transcription of specific sets of early response genes.

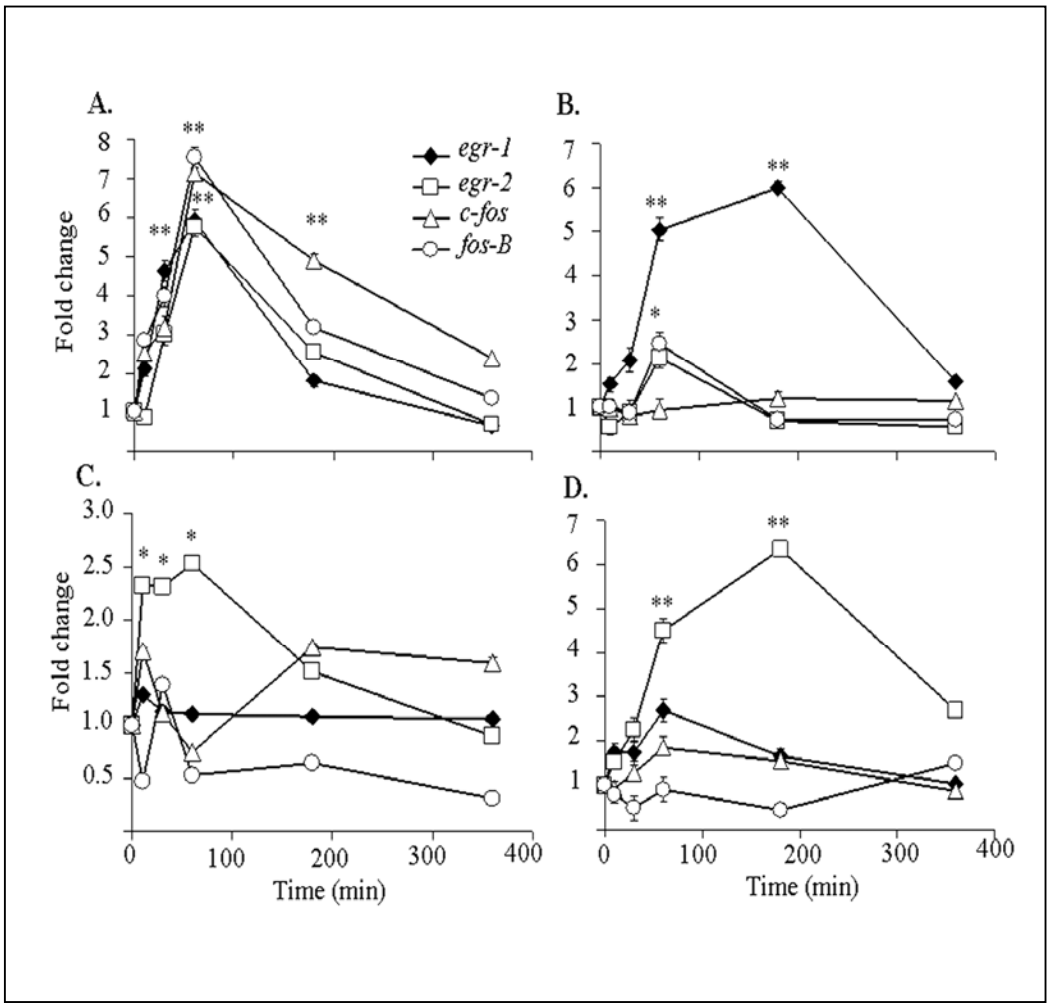

Figure 6.1. GDNF and NTN regulated different early response genes in GFRα1a and GFRα1b expressing cells. Neuro2A cells stably expressing GFRα1a or GFRα1b were seeded sub-confluent on 10% FBS media overnight, and were then serum-deprived with 0.5% FBS media for another night. Cells were then stimulated with GDNF (50 ng/ml) or NTN (50 ng/ml), for time period as indicated. Fold change of mRNA expressions of early response genes in Neuro2A cells expressing GFRα1a stimulated with GDNF (**A**) or NTN (**B**), or cells expressing GFRα1b stimulated with GDNF (**C**) or NTN (**D**) were measured by quantitative real time PCR. Similar results were repeated in four individual experiments. Error bars indicate standard deviations of triplicate measurements. Significant differences in expression of genes between ligand stimulated and control samples were calculated using paired Students t-test. A value of $P<0.05$ was considered significant (**$P<0.001$, *$P<0.05$).

6.2.2 GFRα1a but not GFRα1b induced MAPK dependent-neurite outgrowth upon ligand stimulations

Using Neuro2A cells stably expressing GFRα1a or GFRα1b, neuritogenesis mediated by GDNF and NTN was investigated. Startling differences in neurite outgrowth mediated by GFRα1 isoforms were observed. GFRα1a but not GFRα1b induced neurite outgrowth when stimulated with ligand (Fig. 6.2). GDNF or NTN stimulated GFRα1a expressing cells to extend neurites with at least twice the body length and the number of cells bearing neurites was about 30%, comparable to that induced by retinoic acid (Fig. 6.2). In contrast, when stimulated with ligands, cells expressing GFRα1b remained round in shape and looked similar to naïve neuroblast in morphology (Fig. 6.2A). Retinoic acid was used as a positive control for inducing neurite extension and transfectants with either, GFRα1a or GFRα1b, showed similar extent of neurite outgrowths. This is indicative of the similar neurite outgrowth capabilities of these cells.

The activation of ERK1/2 in both GFRα1 isoforms when treated with GDNF and NTN has previously been shown (Yoong *et al.*, 2005). Here, it is further demonstrated that ligand induced neurite extension of GFRα1a cells is MAPK dependent, as this morphological change was sensitive to the MAPK inhibitor, U0126 (Fig. 6.2B). The concentration of U0126 (5 μM) had no significant effect on the survival of Neuro2A transfectant (data not shown).

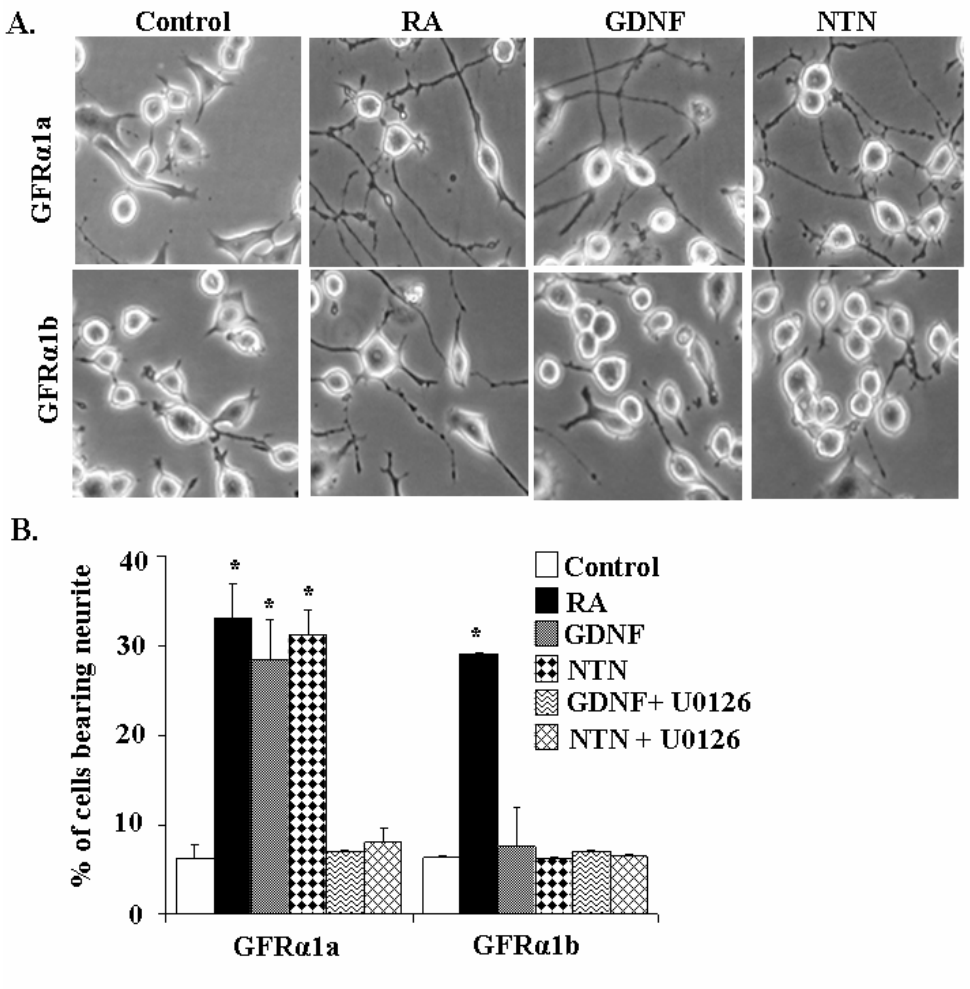

Figure 6.2. GFRα1 isoforms mediated distinct neuritogenic activities. Neuro2A cells stably expressing GFRα1a or GFRα1b were treated in 0.5% serum media, with or without GDNF or NTN (50 ng/ml) for three days. Retinoic acid (RA) (5 μM) was used as a positive control to differentiate cells. **A,** Digital phase contrast images (magnification x200) of Neuro2A cells stably expressing GFRα1a or GFRα1b, treated for three days with RA, GDNF or NTN. **B,** Percentages of cells bearing neurite were counted for cells bearing neurite twice longer than cells bodies. Effects of MEK1/2 inhibitor, U0126 (5 μM) on GDNF and NTN induced neurite outgrowth were also investigated. Experiments have been repeated twice in three individual clones, with similar results. Significant differences in percentage of cells bearing neurite between ligand stimulated and control samples were calculated using paired Students t-test (**P<0.002).

To further understand the morphological changes of ligand activated GFRα1 isoforms, the distribution of two neuronal-cytoskeletal components in the differentiated Neuro2A transfectants were investigated. When treated with GDNF (or NTN), Neuro2A cells stably expressing GFRα1a extended long, axonal neurites, which were doubly-stained for NF-H and F-Actin (Fig. 6.3). At the extremities of the neurites are growth cones that were stained positive for F-Actin. Stimulation with either GDNF or NTN did not induce significant neurite extension in GFRα1 cells and F-Actin staining at the peripheral of cells was obvious (Fig. 6.3). These results further confirmed the distinct neuritogenic activities of the GFRα1 isoforms.

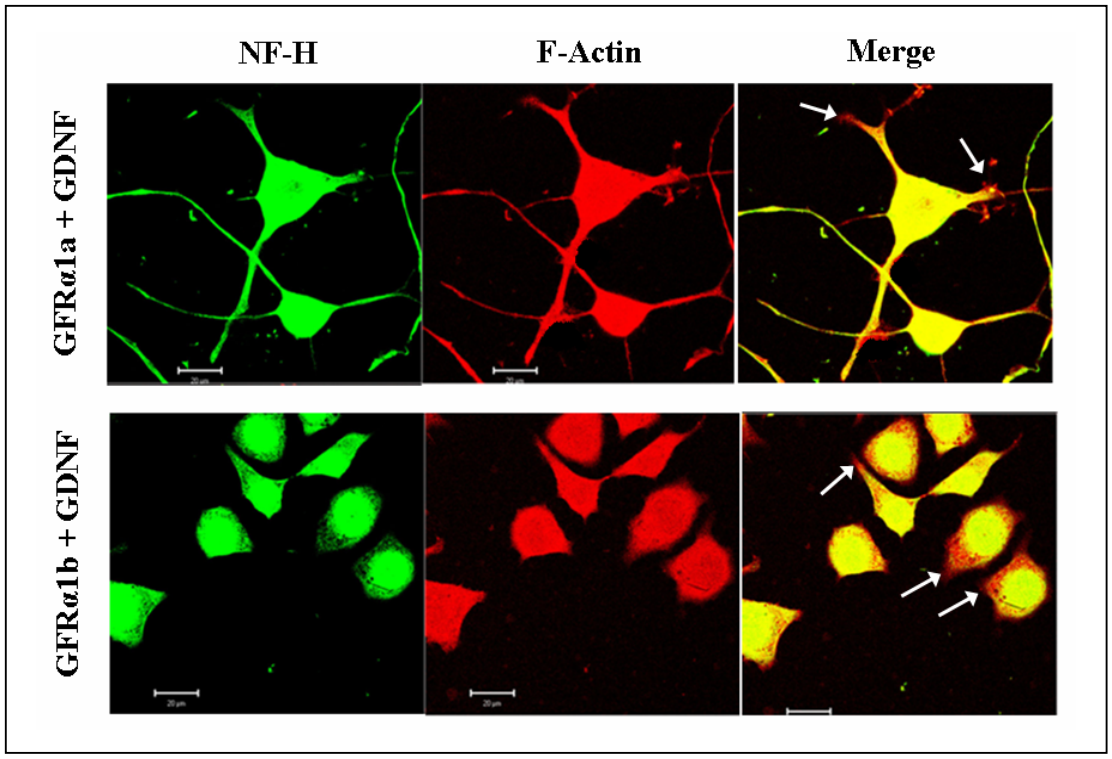

Figure 6.3. Confocal images for double staining of heavy chain neurofilament (NH-F) and F-Actin in GFRα1a or GFRα1b treated with GDNF. Neuro2A cells stably expressing GFRα1a or GFRα1b seeded on chamber slides were treated with GDNF (50 ng/ml) for three days. Cells were then fixed with paraformaldehyde and double-stained for NF-H (green) and F-actin (red). In GFRα1a cells, double immunostaining reveals the co-localization (yellow) of cytoskeletal components, NF-H and F-Actin, along the neurites, while only F-Actin was stained at the growth cones (arrow). In GFRα1b cells, F-Actin stained the peripheral of cells, with no neurite extension observed. Scale bars, 20 μm.

6.2.3 GFRα1b inhibited ligand induced neuritogenic activities of GFRα1a in a RhoA-ROCK dependent mechanism

The lack of morphological changes when GFRα1b was stimulated by ligands may have two possibilities. One is that ligand activated GFRα1b is *NOT* involved in the process of neurite extension, or, that GFRα1b may act similarly to GFRα2b, actively inhibiting some stimuli induced neurite outgrowths (Chapter 5).

In order to test the hypothesis that GFRα1b may inhibit the process of neurite extension by ligand activated GFRα1a, both receptor isoforms were co-expressed in Neuro2A cells. Using pIRES bi-cistronic system, GFRα1b was expressed in the 5' multiple cloning site (MCS) A while GFRα1a was expressed in 3' cloning site (MCS-B). Interestingly, no neurite extension was observed in the GFRα1b and GFRα1a co-expressing cells upon ligand stimulations (Fig. 6.4A). As controls, clones with GFRα1a expressed in MCS-B alone or GFRα1b expressed in MCS-A alone were generated (Fig. 6.4B). When stimulated with ligands, these control clones showed morphological changes (Fig. 6.4) recapitulating the changes observed with the pIRES-neo system (Fig. 6.2). With all the transfectants, retinoic acid induced neurite outgrowths, indicative of the capabilities of these cells to extend neurites (Fig. 6.4B). GDNF or NTN inhibited neurite extension in cells co-expressing GFRα1a and GFRα1b (Fig. 6.4). These observations are consistent with the idea that the activation of GFRα1b antagonizes ligands induced neuritogenic activities of GFRα1a.

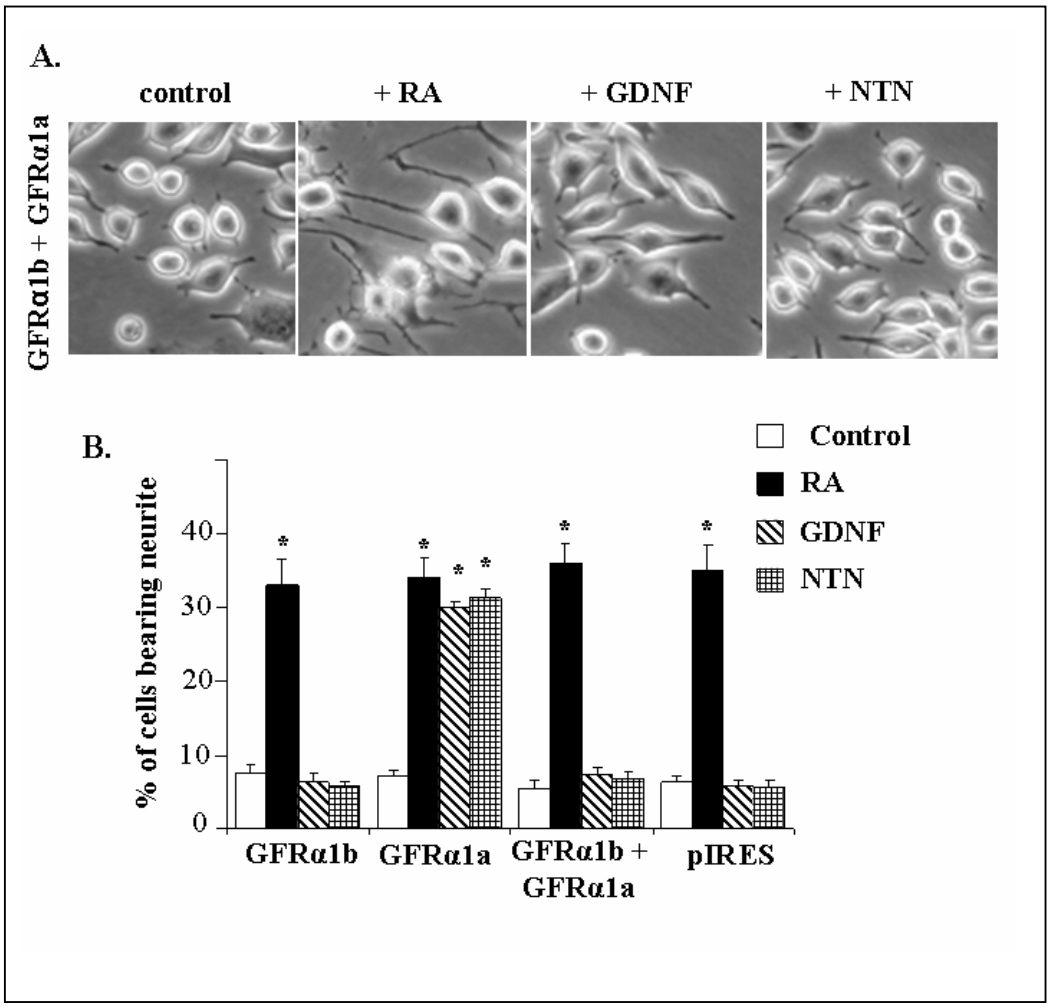

Figure 6.4. GFRα1b attenuated ligand induced neurite outgrowth in GFRα1a when co-expressed. A, Digital phase contrast images (magnification x200) of Neuro2A cells stably co-expressing GFRα1a with GFRα1b (GFRα1a + GFRα1b), treated with 0.5% FBS media with or without 5 μM retinoic acid (RA), GDNF or NTN (50 ng/ml). **B**, Differentiation profiles of cells stably expressing GFRα1a (expressed at 5' multiple cloning site, MCS-A), GFRα1b (expressed at 3' multiple cloning site, MCS-B), GFRα1a with GFRα1b, or bi-cistronic vector control (pIRES). Percentages of cells bearing neurite were counted for cells bearing neurite twice longer than cells bodies. Experiments were repeated twice in two individual clones, with similar results. Significant differences in percentage of cells bearing neurites between ligand stimulated and control samples were calculated using paired Students t-test (*P<0.002).

In the previous study, GFRα2b was found to inhibit neurite extension by the RhoGTPase mechanism (Chapter 5). It is possible that GFRα1b may also regulate the process of neurite outgrowth inhibition by a similar mechanism. The role of

RhoGTPases in GFRα1b inhibitory activities was investigated using a Rho antagonist, exoenzyme C3 transferase. As shown in Fig. 6.5, significant neurite extension was observed in GFRα1b and GFRα1a co-expressing cells when exoenzyme C3 transferase was added along with GDNF ligands. These results indicate that GFRα1b inhibition on ligand induced neurite outgrowths of GFRα1a is abolished in the presence of C3 transferase, suggesting that the inhibitory activity of GFRα1b is Rho dependent. Using a ROCK inhibitor, Y27632, the extent of neurite outgrowth in GFRα1 isoforms co-expressing cells was increased. These suggest that ROCK, a downstream effector of RhoA, may also be involved in the neurite outgrowth inhibitory effects of GFRα1b.

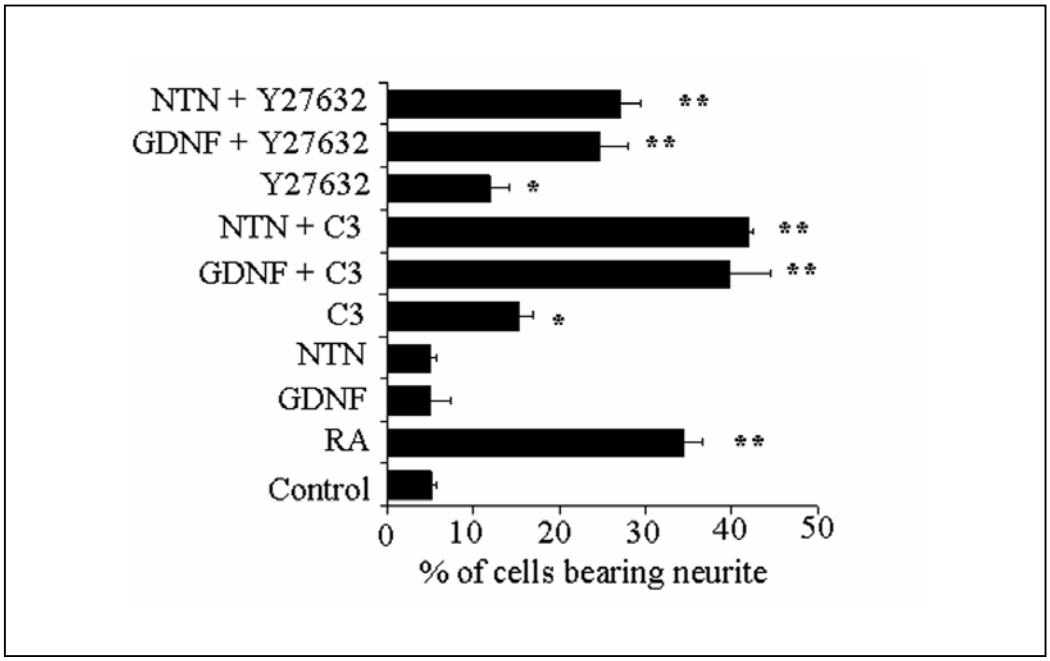

Figure 6.5. GFRα1b attenuated ligand induced neurite outgrowth of GFRα1a, in a Rho-ROCK dependent mechanism. Effects of Rho antagonist, exoenzyme C3 transferase (1 μg/ml) and ROCK inhibitor, Y27632 (10 μM) on GFRα1b attenuation of ligand induced neurite outgrowth of GFRα1a. Neuro2A cells stably co-expressing GFRα1a with GFRα1b were stimulated with retinoic acid (RA), GDNF, or NTN, with or without exoenzyme C3 transferase or Y27632. Percentages of cells bearing neurite were counted for cells bearing neurite twice longer than cells bodies. Experiments were repeated twice in two individual clones, with similar results. Significant differences in percentage of cells bearing neurites between ligand stimulated and control samples were calculated using paired Students t-test (*$P<0.05$, **$P<0.002$).

To further confirm the involvement of RhoA in GFRα1b inhibitory activities, the effect of RhoA dominant negative mutant (RhoA-DN) was investigated next (Fig. 6.6).

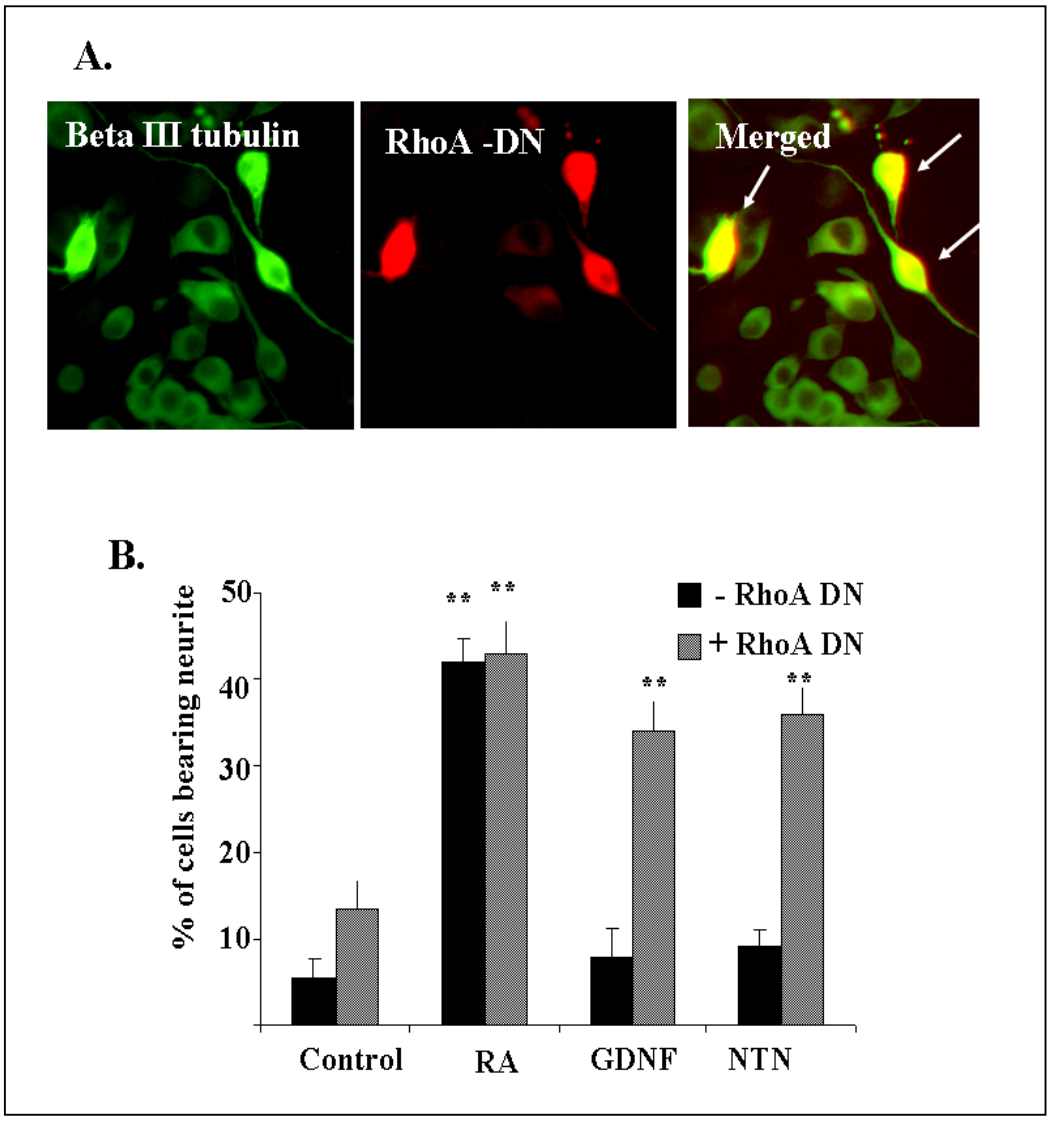

Figure 6.6. RhoA dominant negative mutant prevented inhibitory effects of GFRα1b. A, GFRα1a and GFRα1b co-expressing cells transfected with HA-tagged RhoA dominant negative mutant (RhoA-DN) were differentiated with RA in the presence of GDNF. Cells were stained with beta III tubulin (green) and anti-HA (red). Cells double-stained with beta III tubulin and HA-RhoA-DN showed positive for neurite outgrowth (arrow head). (magnification x200) **B,** Cells count for neurite outgrowth mediated by GFRα1a and GFRα1b co-expressing cells treated with or without RhoA DN. Significant differences in percentage of cells bearing neurites were calculated between ligand stimulated and control samples, using paired Students t-test (**P≤0.01).

Expression of RhoA-DN in GFRα1b cells resulted in the increase of the percentage of cells bearing neurite when stimulated with either GDNF or NTN (Fig. 6.6B). Neurite outgrowths were observed in cells stained positive for HA-tagged RhoA-DN and for beta III tubulin (Fig. 6.6A). These results further indicate the involvement and importance of RhoA in the inhibitory activities of GFRα1b.

The combinatory effects of GDNF or NTN and retinoic acid on neurite extension in GFRα1a or GFRα1b expressing cells was further examined. Extensive neurite extension was observed in retinoic acid treated GFRα1a and GFRα1b expressing cells even in the presence of either GDNF or NTN (Fig. 6.7A, B). Thus, GFRα1b did not inhibit retinoic acid induced neurite outgrowth.

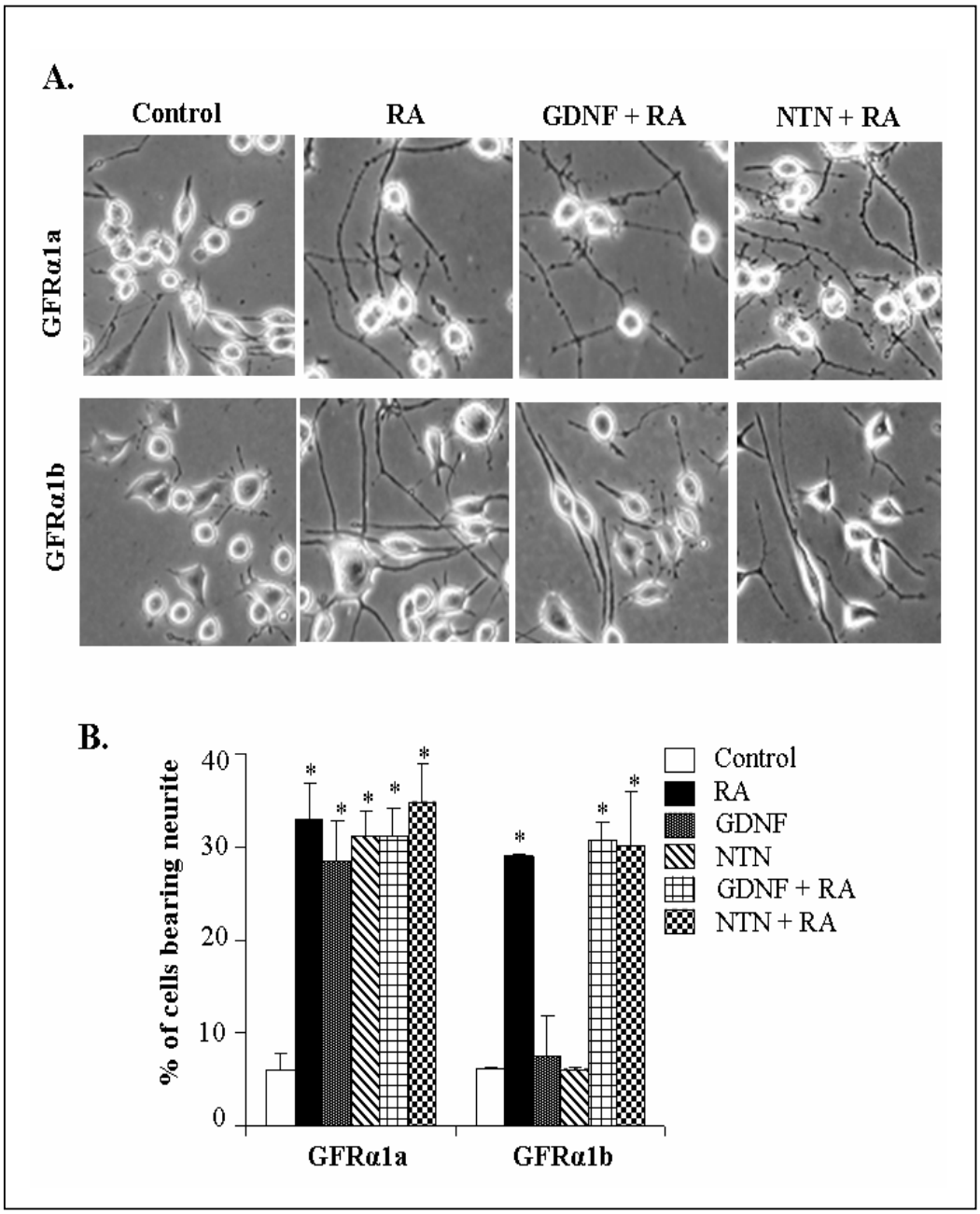

Figure 6.7. Combinatory effect of retinoic acid and GDNF ligands on neuritogenic activities of GFRα1 isoforms. Neuro2A cells stably expressing GFRα1a or GFRα1b were treated with 0.5% FBS media with or without 5 μM Retinoic Acid (RA), or a combination of RA with GDNF or NTN (50 ng/ml). **A,** Digital phase contrast images (magnification x200) of Neuro2A cells stably expressing GFRα1a or GFRα1b, treated with RA, or RA in combination with GDNF or NTN. **B,** Percentages of cells bearing neurite were counted for cells bearing neurite twice longer than cells bodies. Experiments were repeated twice in three separate clones, with similar results. Significant differences in percentage of cells bearing neurite between ligand stimulated and control samples were calculated using paired Students t-test (*P<0.002).

6.2.4 Differential regulation of GFRα1 and Ret isoforms expression in retinoic acid differentiation of mouse embryonic stem cells

As GFRα1b can regulate the neuritogenic activity of GFRα1a, the expressions of these isoforms may be differentially regulated in neurodevelopment. To gain an insight into this possibility, we examined the expression profiles of these GFRα1 and Ret isoforms in neuronal differentiation of mouse embryonic stem cells (mESC) using retinoic acid (Bain *et al.*, 1995; Bibel *et al.*, 2004). Briefly, embryoid bodies, containing neural precursors, were generated by culturing mESC in serum reduced media supplemented with leukemia inducing factor (LIF) for four days, and exposure to retinoic acid for a further four days. Neuronal precursor cells obtained using this induction strategy have been characterized and some neurotrophic factor receptors, including the GFRα1 and Ret were shown to be up-regulated (Lee *et al.*, 2005a). However, the expression profile of the receptor isoforms was not characterized. To extend the findings, the expression profiles of GFRα1, GFRα2 and Ret isoforms in the neuronal precursor cells were quantified, using 2 different mESC cell lines, the AB2.2 and E14 cells.

As shown in Fig. 6.8A, both expressions of GFRα1a and GFRα1b increased after retinoic acid induction (x-axis labeled as EB4-4+RA), with the expression level of GFRα1a higher than that of GFRα1b. Expression of GFRα1a increased by 34.5±1.24 folds in AB2.2 cells, and 22.0±1.78 folds in E14 cells, after retinoic acid induction. On the other hand, expression of GFRα1b also increased by 24±1.03 folds and 30±2.13 folds in AB2.2 and E14 cells, respectively. However, the expression levels of GFRα1a were about six times higher than that of GFRα1b in these neuronal precursor cells derived from AB2.2 and E14 cells.

Ret9 was the dominant isoform expressed in non-differentiated mESC cells. The expression of Ret 9 was about 17 and 5 times higher than Ret51 in non-

differentiated AB2.2 and E14 mESC cells, respectively (Fig. 6.8B). However, the expression of Ret51 was significantly up-regulated in retinoic acid-induced neuronal precursor cells and was the dominant isoforms expressed (Fig. 6.8B). The expression of Ret 51 increased 34±2.31 fold in AB2.2, and 45±2.19 fold in E14 cells after retinoic acid induction. The expression of Ret9 isoform did not change significantly on induction of neuronal differentiation in both AB2.2 and E14 cells (Fig. 6.8B). On the other hand, expressions of the GFRα2 isoforms remained low before or after the retinoic acid induced neuronal differentiation of mESC (data not shown). These findings indicate that GFRα1 and Ret isoforms are differentially regulated in neuronal differentiation of mESC. GFRα1a and Ret51 are the major isoforms expressed in neuronal precursor cells induced from mESC, suggesting their potential role in the differentiation and maturation of neuronal precursor cells.

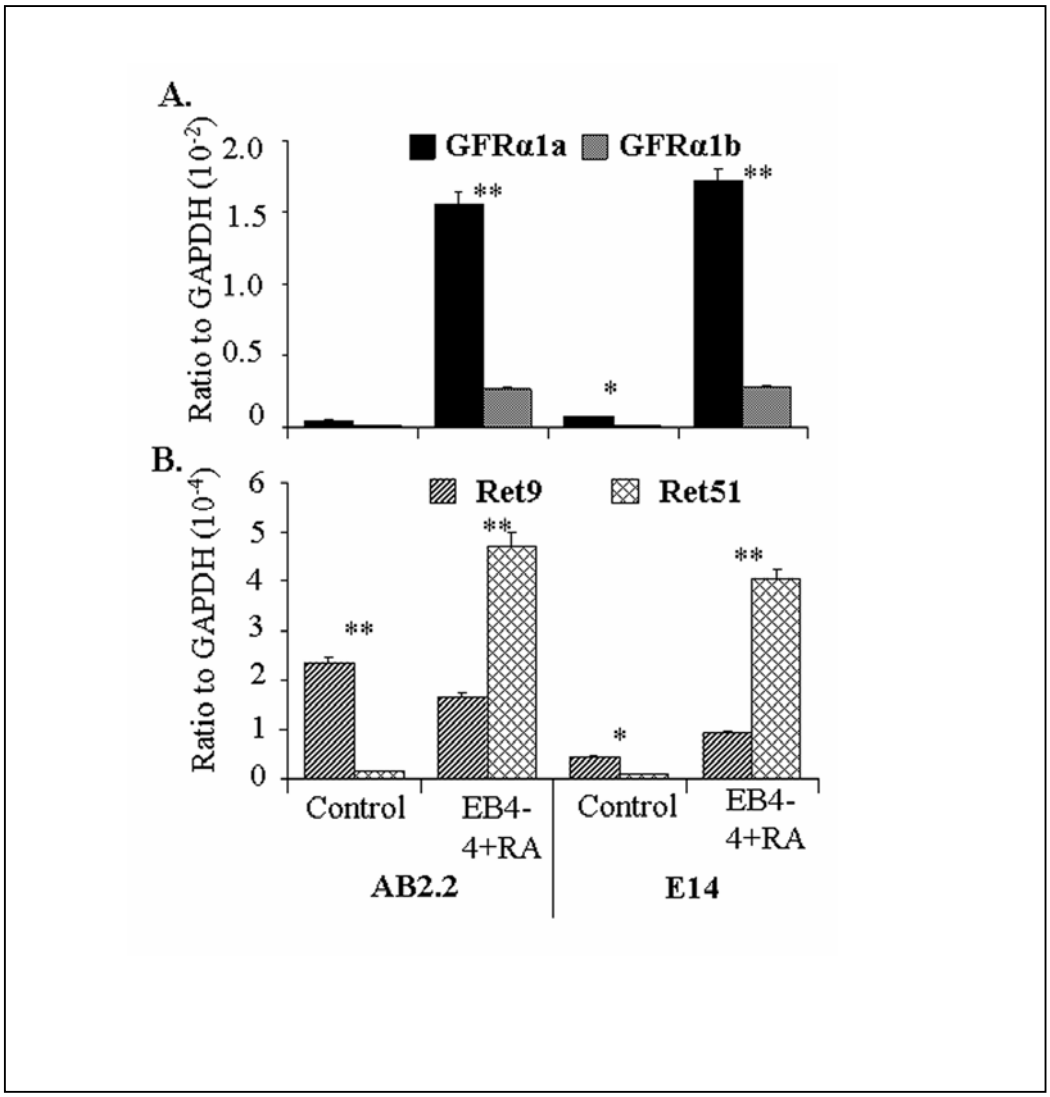

Figure 6.8. Differential regulation of GFRα1 and Ret isoforms gene expression in retinoic acid induced neuronal differentiation of mouse embryonic stem cells. Neuronal precursors of AB2.2 and E14 cells were induced from embryoid bodies using the 4-4+ retinoic acid method. Briefly, embryoid bodies were induced by plating mouse embryonic stem cells, AB2.2 and E14 on non-adherent Petri dish in 10% FBS media (without LIF) for four days and later supplemented with retinoic acid (5 μM) for subsequent four days. Expressions of GFRα1 **(A)** and Ret **(B)** isoforms in control and retinoic acid differentiated (EB4-4+RA) mouse embryonic stem cells were measured by quantitative real time PCR. Error bars indicate standard deviation of 3 measurements. Similar results were repeated in four experiments. Significant differences between expressions of isoforms were calculated using paired Students t-test. A value of $P<0.05$ is considered significant (*$P<0.05$, **$P<0.01$).

6.3 Discussion

This study provides the first evidence of the distinct neuritogenic activities of GFRα1 isoforms and the novel inhibitory activity of GFRα1b isoform in antagonizing neurite outgrowth mediated by ligand activated GFRα1a.

GFRα1 isoforms regulate the expression of distinct immediate early response genes when stimulated by either GDNF or NTN. This observation extends the previously reported distinct biochemical activities and gene expression profiles of activated GFRα1 isoforms (Charlet-Berguerand *et al.*, 2004; Yoong *et al.*, 2005). GFRα1a regulated *egr-1*, *egr-2*, *c-fos* and *fosB* when stimulated by GDNF, but *egr-1* is only up-regulated when stimulated with NTN. In contrast, GFRα1b only regulated *egr-2* when stimulated by either GDNF or NTN.

The activation of *egr-1* when neuronal cells are differentiated by NGF is well documented (Harada *et al.*, 2001; Levkovitz *et al.*, 2001; Milbrandt, 1987; Sukhatme *et al.*, 1988). *egr-1* is now thought to activate cdk5 and phosphorylate PP1, both are downstream effectors essential for neuronal differentiation (Li *et al.*, 2007). The increase of *egr-1* by ligand stimulated GFRα1a and the increase in neurite outgrowth are consistent with the above observations. Interestingly, *egr-2* but not *egr-1* is activated when GFRα1b is stimulated with either GDNF or NTN. The role of *egr-2* in regulating neuronal morphologies is currently unknown.

When co-expressed, GFRα1b inhibited ligands induced neurite outgrowth mediated by GFRα1a. The inhibitor studies suggest the involvement of Rho and ROCK pathway in the ligands mediated inhibitory activities of GFRα1b. The involvement of RhoA in the inhibitory activity of GFRα1b was further confirmed in this study by using a dominant negative mutant of RhoA. These findings are consistent with a neurite outgrowth inhibitory role of GFRα1b.

This study raised new insights into the existence of a common self-regulatory mechanism in the signaling and neuritogenic activities in GFRα1 and GFRα2. With both receptors, particular splice variants can inhibit the others in inducing neurite outgrowths. However, there are indications that the inhibitory activities and mechanisms of GFRα1b differ from that of GFRα2b. Firstly, ligand activated GFRα2b but not GFRα1b, can inhibit retinoic acid (a non-GFR stimuli) induced neurite outgrowth. Secondly, the data suggest that the inhibitory activities of GFRα2b may involve RhoA, but not its downstream kinase effector, ROCK. In contrast, the GFRα1b inhibitory activities appear to involve both RhoA and ROCK. Both GFRα1 and GFRα2 transduce downstream signaling via recruitment and activation of the tyrosine kinase co-receptor, c-Ret. Understanding the specificity of GFRα1b and GFRα2b in activating different tyrosine residues of c-Ret (Plaza-Menacho *et al.*, 2006) may provide clues to the downstream mechanisms involved in their inhibitory activities.

It has previously been shown that expressions of GFRα1 and Ret are up-regulated during the neuronal differentiation of mESC (Lee *et al.*, 2005a). However, the expression levels of the specific isoforms were unknown. In this study, the expression levels of the spliced variants of GFRα1 and Ret were accurately quantified by real-time PCR. The high expression of GFRα1a but not GFRα1b in retinoic acid differentiated mESC suggests a possible role of GFRα1a in regulating neuronal differentiation. The dominant isoform of Ret expressed in mESC changed from Ret9 in naïve mESC to Ret51 in retinoic acid differentiated mESC. Mutagenesis study has shown that Ret9 but not Ret51 is vital for the development of kidney and enteric nervous system, and that the Ret51-deficient mouse is both viable and normal (de Graaff *et al.*, 2001). While the functions of Ret in kidney formation and development

are just beginning to be elucidated, the mechanisms and role/s of Ret isoforms in neuronal development is presently unclear.

The existence of multiple isoforms is common in GDNF family receptors. Alternatively spliced isoforms have been reported in GFRα1, GFRα2, and GFRα4 (see Literature Reviews, Chapter 2). These studies have shown that the truncated isoforms of GFRα1 (GFRα1b) and GFRα2 (GFRα2b) exert similar yet distinct inhibitory and anti-neuritogenic effects. The current results suggest that alternative splicing may be a common mechanism by which GFRα genes generate isoforms with opposing neuritogenic activities. Further studies are warranted to understand the physiological role of GFRα1b and GFRα2b in GDNF signaling and neuronal functions.

Chapter 7 Part V: Neuritogenic mechanisms of GFRα2a and GFRα2c

7.1 Background and objectives

Neurite outgrowth is a complex differentiation process stimulated by many neuronal growth factors, transmitters, electrical activity and influenced by the extracellular cues. The outgrowth process is critically dependent on signaling which reorganizes the actin cytoskeleton by regulation of many actin-interacting proteins functioning downstream of Rho-family GTPases (Zhou and Cohan, 2004). In addition, *de novo* synthesis of numerous proteins is known to occur during neuronal differentiation (da Silva and Dotti, 2002; Gallo and Letourneau, 2004; Tojima and Ito, 2004). Extending the search for biomolecules involved in neurite outgrowths, a large-scale RNAi screen identified more than 100 genes to be involved in synapse structure and function (Sieburth *et al.*, 2005) and a microscopy-based approach has identified several novel proteins involved in the neurite outgrowth process (Laketa *et al.*, 2007). Much is still to learn about the precise and tight control of genes and proteins involved in the temporal events in neurite outgrowths.

Ligand activated GFRα2a and GFRα2c induce neurite outgrowths in Neuro2A cells (Chapter 4). GFRα2c lacks the exon 2 and 3, generating a protein with deletion of 132 amino acids, as compared to the full-length receptor isoforms, GFRα2a. Despite the vast differences in N-terminal structure, both GFRα2a and GFRα2c isoforms induced neurite outgrowths in a ligand dependent manner. However, there are significant differences in AKT signaling and the regulation of the expressions of some immediate early response genes, thus, indicating that GFRα2a and GFRα2c may utilize distinct mechanism/s in mediating ligand induced neurite outgrowths (Chapter 4). In this study, we examine the process of neurite outgrowths induced by these two receptor isoforms in greater detail.

In this chapter, effects of kinase inhibitors on ligand activated neurite outgrowth in GFRα2a and GFRα2c transfected were investigated. We further compare the effects of ligands withdrawal in neurite outgrowth mediated by these two GFRα2 isoforms. Expressions of various neuronal markers in ligand induced neurite outgrowth of these isoforms were also measured and compared.

7.2 Results

7.2.1 Ligand activated GFRα2a and GFRα2c mediated neurite outgrowths via distinct signaling pathways

We first explored the involvements of various signaling kinases in neurite outgrowths in GFRα2a and GFRα2c cells by using selective inhibitors. The differential effects of kinase inhibitor on ligand induced neurite outgrowth in GFRα2a and GFRα2c cells are shown in Fig. 7.1. The Src inhibitor, PP2 (2 μM) attenuated ligand induced neurite outgrowths in both GFRα2a and GFRα2c cells (Fig. 7.1A). On the other hand, MEK1/2 inhibitor, U0126 (5 μM) attenuated ligand induced neurite outgrowths in GFRα2a cells, but not in GFRα2c cells (Fig. 7.1B). A PLCγ inhibitor, U73122 (0.1 μM) also exhibited similar effects, where it only attenuated ligand induced neurite outgrowth in GFRα2a cells, but not in GFRα2c cells (Fig. 7.1C). The concentrations of kinase inhibitors used in the studies have no significant effects on cells viability (data not shown). These results indicate that different signaling mechanisms are involved in ligand induced neurite outgrowth of GFRα2a and GFRα2c.

Other inhibitors, SB 203580 (p38 inhibitor) and LY 294002 (PI3 kinase inhibitor) had no effect on ligand induced neurite outgrowth by GFRα2 isoforms (data not shown).

Both GFRα2a and GFRα2c have been shown to mediate MAPK activations in Neuro2A cells (Fig. 4.5, Fig. 4.6). The effects of these kinase inhibitors on ligand induced MAPK activations in GFRα2a and GFRα2c cells were further elucidated. The dose effects of different kinase inhibitors on phosphorylations of ERK1/2 were shown in Fig. 7.2. Src kinase inhibitor, PP2 (1, 2, and 10 μM) prevented phosphorylation of ERK1/2 in both ligand induced GFRα2a and GFRα2c cells (Fig. 7.1A). PP3, an

inactive analog of PP2, showed no effect on ERK1/2 activations (Fig. 7.1A). These data suggest that Src is an upstream effector in ligand induced MAPK signaling of GFRα2a and GFRα2c receptor isoforms.

MEK1/2 inhibitor, U0126 (1, 5, and 10 μM), inhibited ligand induced ERK1/2 phosphorylations in both GFRα2a and GFRα2c cells. It is worthy to note that while U0126 inhibited ERK1/2 activation in GFRα2c (Fig. 7.2B), it did not affect ligand induced neurite outgrowth of GFRα2c cells (Fig. 7.1B). These results indicated that Src, but not ERK1/2, is necessary for ligand induced neurite outgrowth in GFRα2c cells.

The PLCγ inhibitor, U73122 (0.05 to 1 μM), showed a dose dependent inhibitory effect on ERK1/2 phosphorylation. Low dose of U73122 (0.1 μM) was used in the neurite outgrowth studies (Fig. 7.1C), as the higher doses (0.5 and 1 μM) significantly affected cells viability. U73122 at 0.1 μM strongly inhibited the ligand induced neurite outgrowth in GFRα2a cells. The same concentration of U73122 did not completely abolish EKR1/2 phosphorylation. This is indicative of the involvement of PLCγ signaling in the outgrowth process and may not involve the activation of ERK1/2.

In conclusion, the results showed that the signaling mechanisms involved in the neurite outgrowths of ligand activated GFRα2a and GFRα2c are sufficiently different.

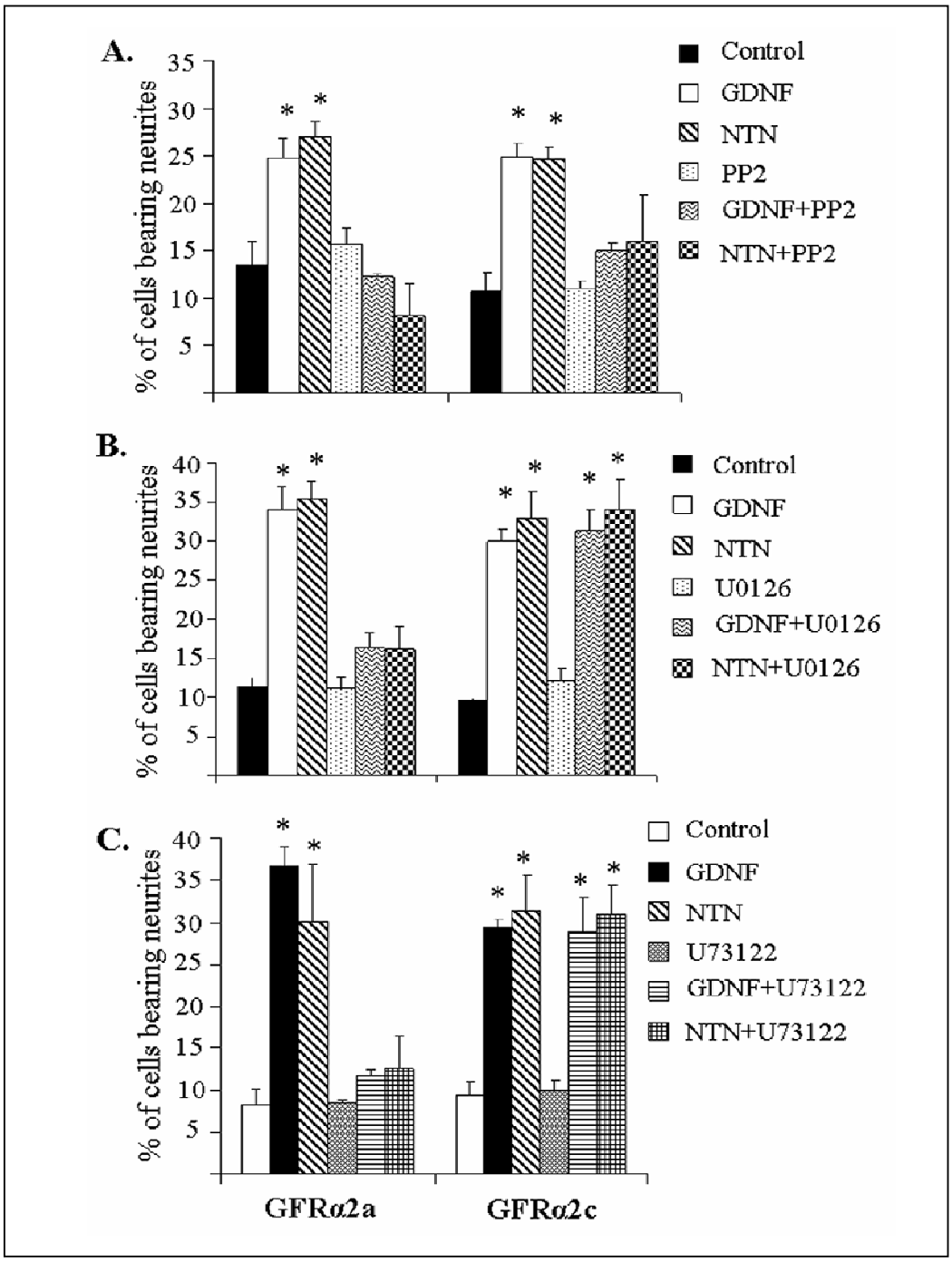

Figure 7.1. Effects of kinase inhibitors on ligand induced neurite outgrowth in GFRα2a or GFRα2c transfected Neuro2A cells. Neuro2A cells expressing GFRα2a or GFRα2c were treated with GDNF or NTN, with or without Src inhibitor PP2 (2 μM) (**A**), MEK1/2 inhibitor U0126 (5 μM) (**B**), or PLCγ inhibitor U73122 (0.1 μM) (**C**). After three days in culture, percentages of cells bearing neurite of at least twice the length of the cell bodies were scored. Similar results were obtained from two individual clones and the experiment was repeated at least twice. Significant differences between ligand stimulated and control samples were calculated using paired Students t-test. A value of $P<0.05$ was considered significant (*$P<0.02$).

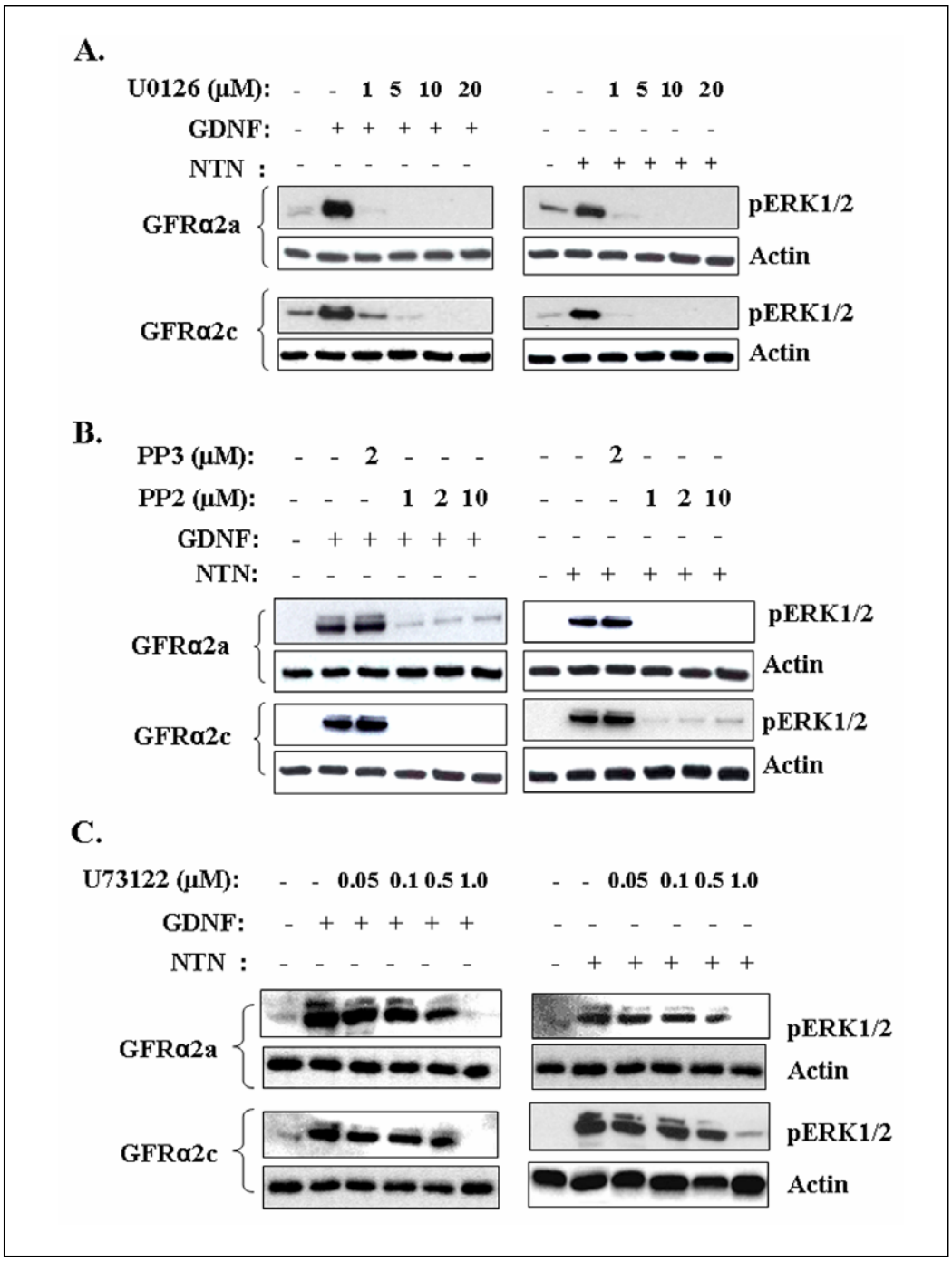

Figure 7.2. Effects of kinase inhibitors on ERK1/2 activation in GFRα2a and GFRα2c cells. Western blots of GFRα2a and GFRα2c cells treated with GDNF or NTN (50 ng/ml) for 10 minutes, with or without Src inhibitor PP2 (**A**), MEK1/2 inhibitor U0126 (**B**), or PLCγ inhibitor U73122 (**C**), at concentrations as indicated. Cells were treated for respective inhibitors for 20 minutes prior to treatment of ligands. In Src inhibitor studies, effects of PP3, an inactive homolog of PP2, were also investigated. After immunoblotted for Phospho-ERK1/2 (pERK1/2), blots were stripped and reprobed for Actin as loading control. Similar results were obtained from two individual clones and the experiment was repeated at least twice.

7.2.2 Withdrawals of ligands produced different effects on neurite outgrowth mediated by GFRα2a and GFRα2c receptor isoforms

All previous neurite outgrowth experiments were studied by treating cells with stimuli for three continuous days in culture. To gain a further insight into the mechanism involved in ligand induced neurite outgrowth in GFRα2 isoforms, the effects of ligand withdrawal at the early time points were closely examined.

The effect of retinoic acid withdrawal in differentiation of naive or pIRES transfected Neuro2A cells was first investigated. Cells were either exposed to retinoic acid continuously for 48 h or for only 10, 30 or 60 min. Significant number of differentiated cells was observed when retinoic acid was present throughout the 48 h (Fig. 7.3). However, no differentiation was observed with cells exposed to retinoic acid for only 10, 30 or 60 min (Fig. 7.3). These observations indicate that exposure to retinoic acid for an hour or less is insufficient to induce significant differentiation.

The effect of time of exposure to GDNF and NTN on neurite outgrowths of GFRα2a and GFRα2c cells were examined next. Neurite outgrowth was observed in GFRα2a cells exposed to GDNF or NTN for 30 min, 1 h and 48 h, but not with cells exposed to the ligands for only 10 minutes (Fig. 7.4A). In contrast, neurite outgrowths were observed with GFRα2c cells exposure to the ligands for 48 h but not those exposed for an hour or less (Fig. 7.4B).

In conclusion, GFRα2a can mediate neurite outgrowth when exposed to the ligand for as short a period as 30 min, while GFRα2c cells required the presence of ligands for longer than 1 hour. It is important to note that the expression levels of the receptors were comparable (Chapter 4). Taken together, GFRα2a and GFRα2c isoforms have different kinetic responses in the process of ligand induced neurite outgrowth.

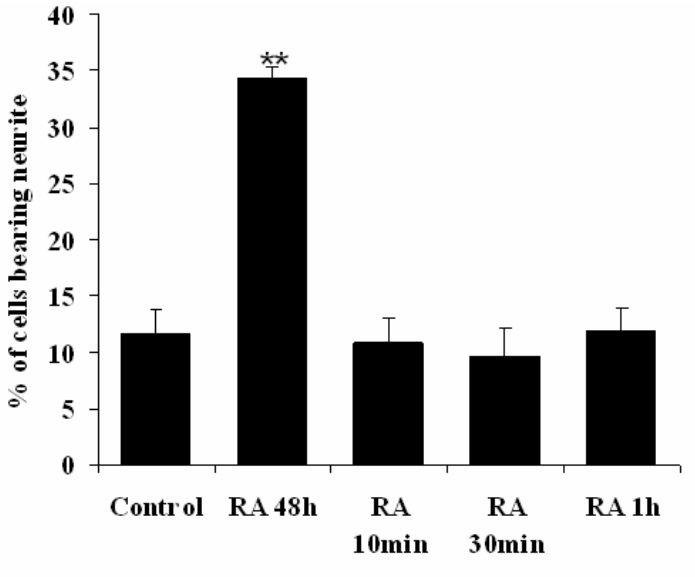

Figure 7.3. Time of exposure of cells to retinoic acid and differentiation. Neuro2A cells were treated with 5 μM of retinoic acid (RA) for the time indicated. The media containing retinoic acid were then removed and replaced with 0.5% serum containing media. The cells were then incubated for 48 h. Significant level of differentiation was observed with cells exposed to RA continuously for 48 h. but not when the cells were exposed to RA for 10, 30 min or 1 h. Differences in percentage of cells bearing neurite were calculated between ligand stimulated and control samples, using paired Students t-test (**P≤0.01). Similar results were repeated in three individual experiments.

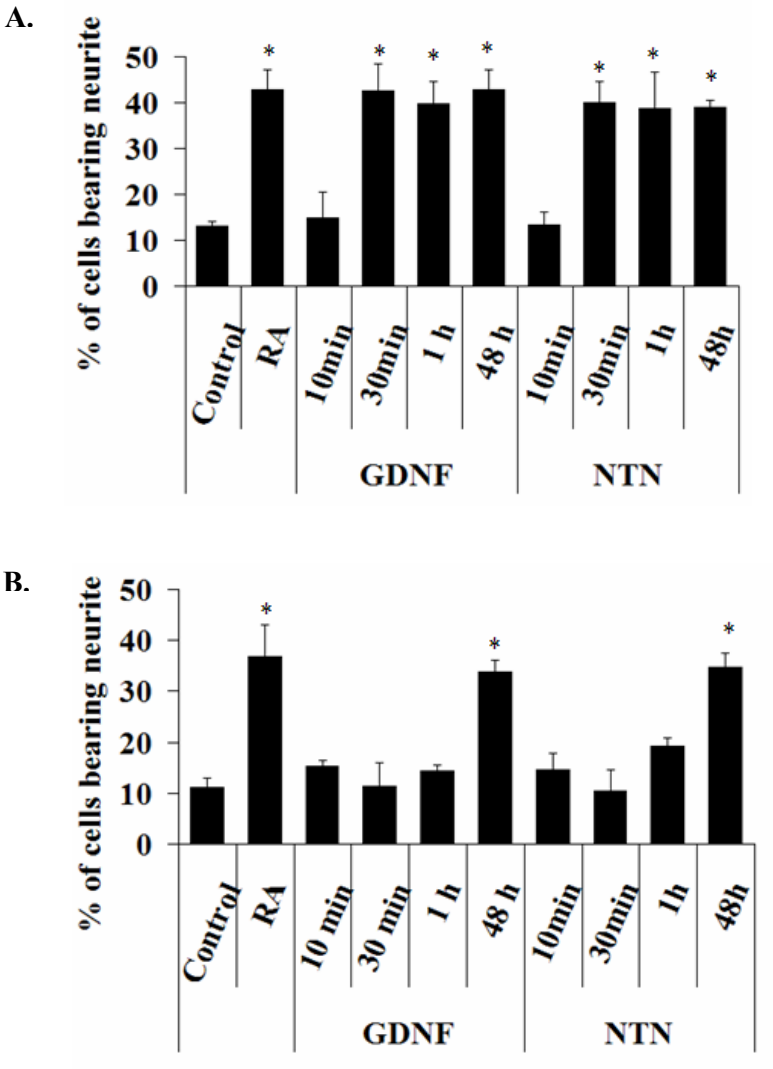

Figure 7.4. Study of ligands withdrawal effects on neurite outgrowth mediated by GFRα2 isoforms in Neuro2A transfectants. Cells were treated with ligand for the time indicated. The differentiation media was then removed, and replaced with only 0.5% serum media. **N**eurite outgrowths of GFRα2a (**A**) and GFRα2c (**B**) cells when exposed to GDNF and NTN for varying periods of time. GDNF or NTN were either present throughout all 48 hours or for only 10, 30 min or 1 h. Differences in percentage of cells bearing neurites were calculated between ligand stimulated and control samples, using paired Students t-test (*P≤0.01). Similar results were obtained in two individual clones with replication of at least three individual experiments.

7.2.3 GFRα2a and GFRα2c share some similar neuronal markers upon ligand induced neurite outgrowth

Collapsin response mediator proteins (CRMP) family comprises five cytoplasmic proteins that have previously been shown to mediate neuronal morphogenesis (Nishimura *et al.*, 2003; Yuasa-Kawada *et al.*, 2003) and are expressed predominantly in developing neuronal systems (Fukada *et al.*, 2000; Wang and Strittmatter, 1996). Although little is known about their precise roles, regulation of CRMP2 by GDNF has been reported (Kodama *et al.*, 2004). However, regulation of other CRMPs family members by GDNF or NTN via GFRα2 receptor is not known. Using quantitative real time PCR, the regulation of *CRMP1B*, *CRMP2A*, *CRMP2B*, *CRMP3*, *CRMP4* and *CRMP5* gene expressions in ligand activated GFRα2a and GFRα2c transfectants were investigated.

Increases in gene expressions of CRMP3 were observed in GDNF and NTN treated GFRα2a and GFRα2c cells (Fig. 7.5). When GFRα2a was stimulated with ligand, CRMP3 expression was up-regulated only at 24 h or later but not at 6 h (Fig. 7.5A). Different kinetics of *CRMP3* expressions were observed in GFRα2c cells, where up-regulation of *CRMP3* was observed as early as 6 h and persisted up to 48 h, with slighter lower level of expression (Fig. 7.5B). No significant change in expression was observed with the other *CRMPs* examined, at 6, 24 or 48 h (data not shown). These results showed that both GFRα2a and GFRα2c when stimulated by ligands, up-regulated the expression of *CRMP3* with different kinetics.

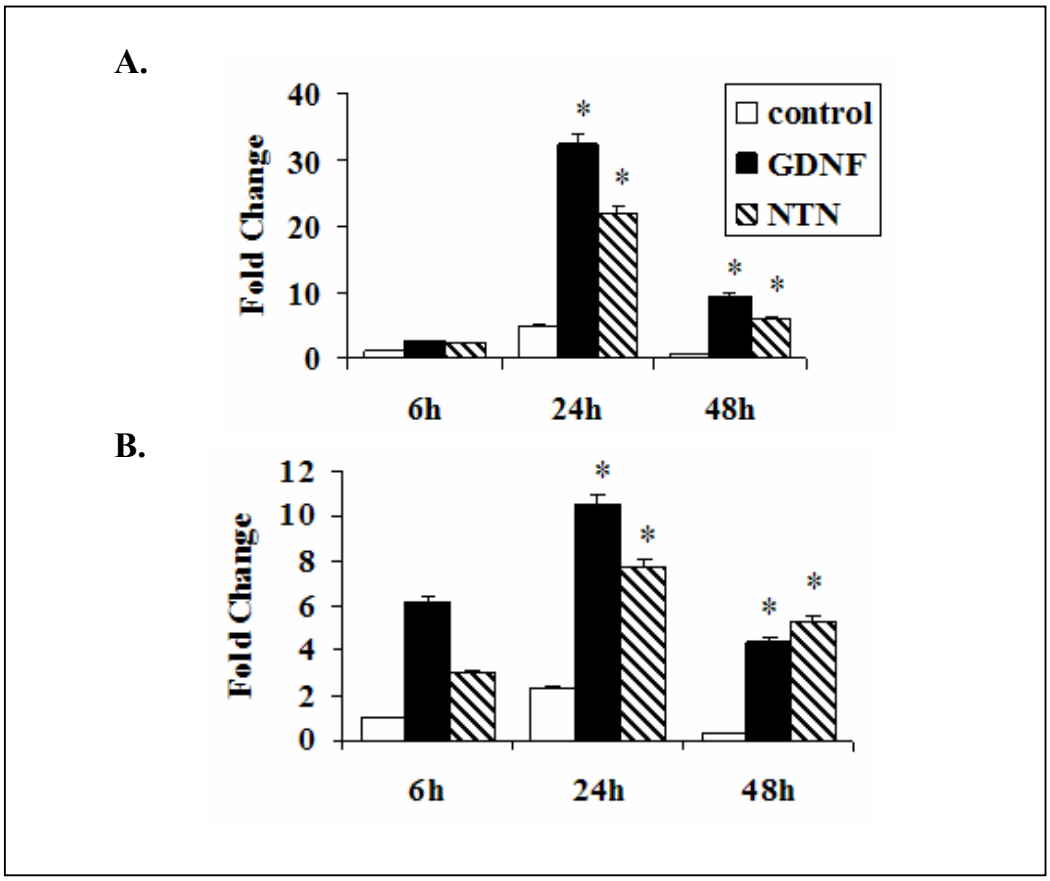

Figure 7.5. Regulation of *CRMP3* gene expression by GFRα2a and GFRα2c, in Neuro2a cells stably expressing these receptor isoforms. Regulation of neuronal marker gene, *CRMP3* (**A, B**) was measured by quantitative real time PCR. Fold change of *CRMP3* gene in GFRα2a (**A**) and GFRα2c (**B**) was measured at 6, 24, and 48 hours after induction of differentiation. Fold change or expression was normalized to expression of respective gene of control at the 6 hour. Significant differences in expression of gene between ligand stimulated and control sample at the 6 hour were calculated using paired Students t-test. A value of $P<0.05$ was considered significant (*$P<0.02$).

Neuro2A cells served as a model to study the selection of cholinergic and GABAnergic cell lineages during differentiation (Manabe *et al.*, 2005). It has been shown that retinoic acid induces cholinergic markers, while dbCAMP induces GABAergic marker in Neuro2A cells, indicating the potential of Neuro2A to adopt cholinergic and GABAnergic characteristics (Manabe *et al.*, 2005). Little is known about the role of GFRα2 receptor in neuronal lineage selection. The expressions of a

cholinergic and GABAergic marker in ligand activated GFRα2a and GFRα2c cells were thus investigated.

Gene expression of a cholinergic marker ChAT was measured at 6, 24 and 48 h of GDNF or NTN treatment in GFRα2a and GFRα2c cells. Expression of ChAT was barely detected in non-induced GFRα2a and GFRα2c transfectants, and no significant up-regulation of ChAT expression was observed after ligand stimulation (data not shown).

The biosynthesis of the inhibitory neurotransmitter, GABA, is catalyzed by two isoforms of glutamate decarboxylase (GAD) proteins, namely GAD_{67} and GAD_{65}, which are encoded by two distinct genes, *GAD1* and *GAD2* respectively. GFRα2a was found to up-regulate the expression of *GAD1* (Fig. 7.6A), but did not affect the expression of *GAD2* (data not shown). On the other hand, GFRα2c showed a different profile in regulating the expressions of these genes. GFRα2c down-regulated the expression of *GAD2* (Fig. 7.6B) and did not affect *GAD1* expression (data not shown).

Interestingly, these results indicate that GFRα2 isoforms may be involved in regulating cell fate and may prefer GABAergic over cholinergic lineage in Neuro2A.

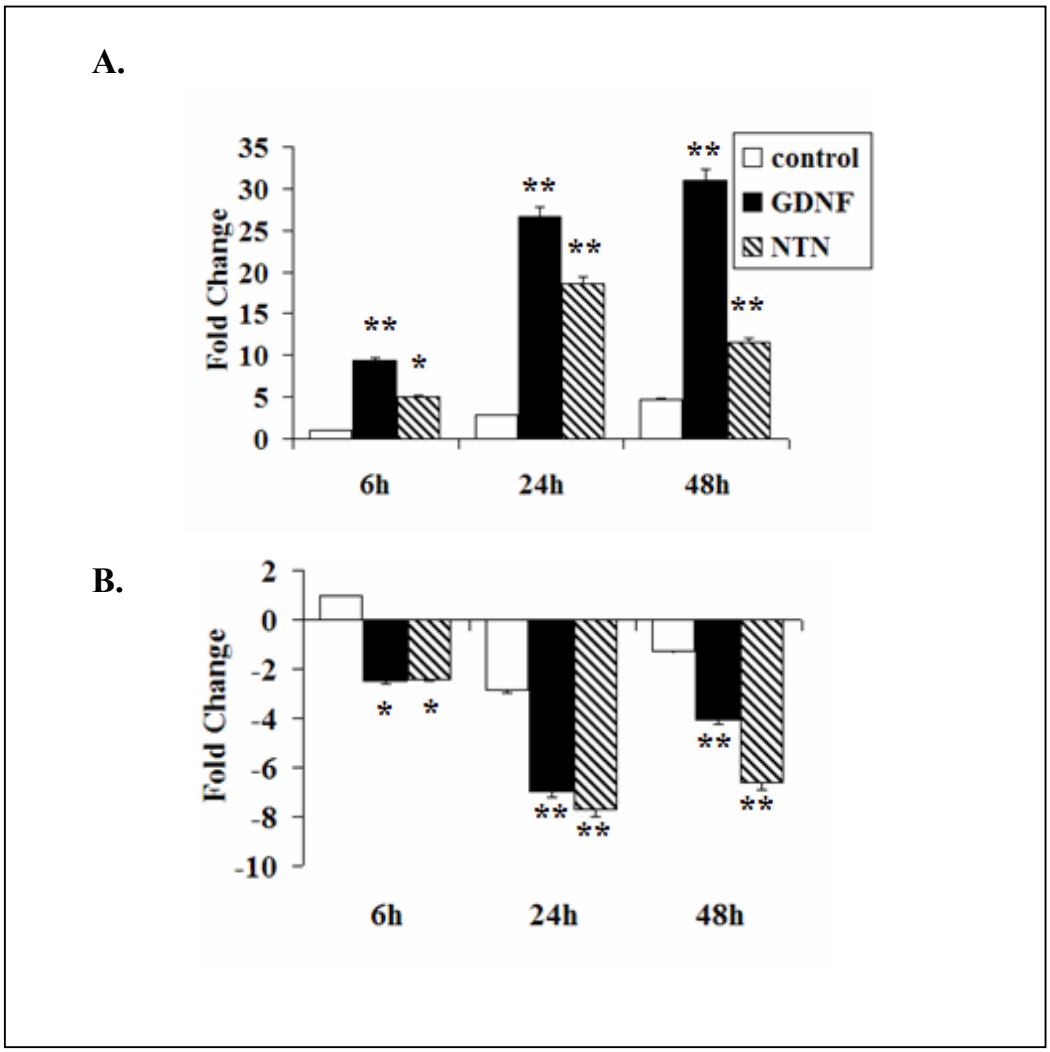

Figure 7.6. Regulation of *GAD1* and *GAD2* expressions by ligand stimulated GFRα2a and GFRα2c. The expression of neuronal markers, *GAD1* and *GAD2*, were measured by quantitative real time PCR. Fold change of *GAD1* gene expression in GFRα2a (**A**) and *GAD2* expression in GFRα2c (**B**) were measured at 6, 24, and 48 h after induction of differentiation. Up-regulation of *GAD1* was detected in GFRα2a cells (**A**) while GFRα2c showed down-regulation of *GAD2* (**B**). Fold change was determined by normalizing the expression of the gene at the designated time points with the respective control sample (unstimulated) at 6 hour. Significant differences in expression of genes between ligand stimulated and control sample at 6 hour were calculated using paired Students t-test. A value of $P<0.05$ was considered significant (*$P<0.05$, **$P<0.01$).

7.3 Discussion

In this study, the biochemical and signaling differences in ligand induced neuritogenic activities of GFRα2a and GFRα2c were studied. While both GFRα2a and GFRα2c induced neurite outgrowths when stimulated with either GDNF or NTN, the underlying neuritogenic mechanisms do not appear to be identical. Studies using various kinase inhibitors revealed different signaling mechanisms to be involved in ligand induced neurite outgrowth of GFRα2a and GFRα2c. Furthermore, the kinetics of exposure to ligands required for neurite outgrowths provide further evidence for different signaling mechanisms used by GFRα2a and GFRα2c. While both GFRα2a and GFRα2c up-regulated the expression of *CRMP3*, the kinetics however differed substantially. Finally, it has also been shown that GFRα2a and GFRα2c up-regulated GABAergic markers in Neuro2A cells differently.

A schematic diagram of the effects of various kinase inhibitors on neurite outgrowths is shown (Fig. 7.7), GFRα2a and GFRα2c utilize distinct signaling pathways in mediating neurite outgrowths. Src, ERK1/2 and PLCγ are intimately involved in neurite outgrowth mediated by GFRα2a and are likely to act cooperatively in neuritogenesis. Neurite outgrowth of GFRα2c was sensitive only to PP2 inhibitor and the inhibitor attenuated ERK1/2 signaling in GFRα2c cells. These observations suggest that neurite outgrowth in GFRα2c cells may be attributed to yet another signaling pathway, which is downstream of Src.

GFRα2a was able to induce neurite outgrowth when exposed to the ligands for as short as 30 minutes while GFRα2c required the presence of ligand for longer than an hour, indicating different kinetics in the signaling mechanisms of these two receptor isoforms. This difference in kinetics is unlikely to be due to the differences in

the expression levels of the GFRα2 isoforms or the co-receptors, NCAM and RET, as these are expressed at comparable levels in all the transfectants (Chapter 4).

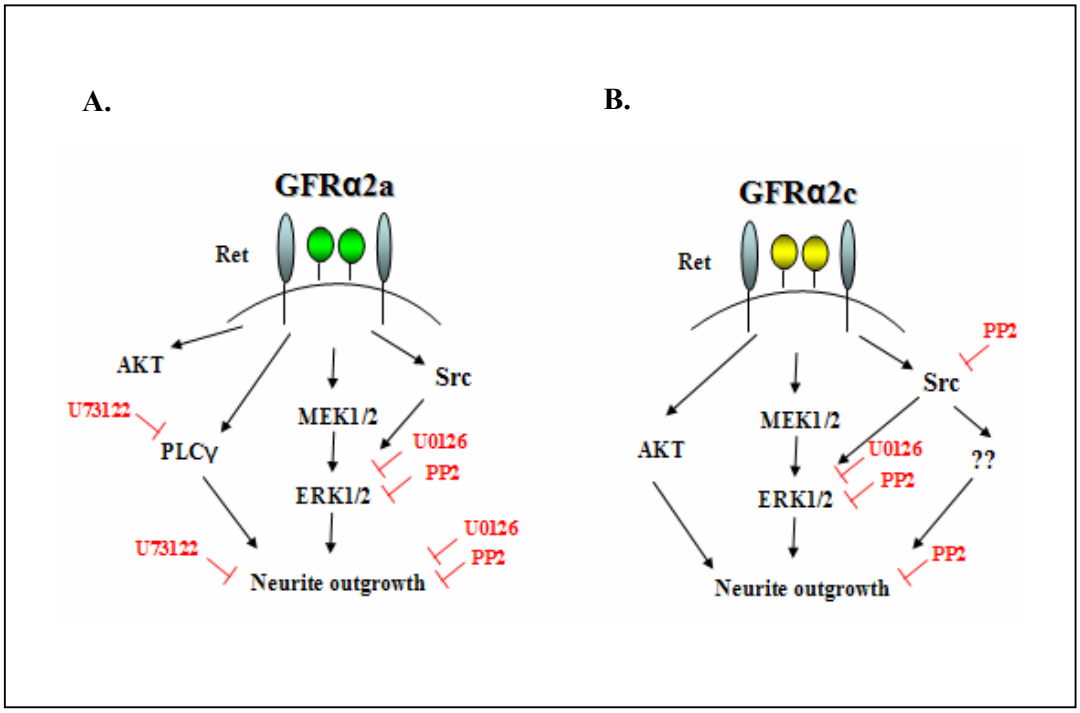

Figure 7.7. Schematic diagram of signaling mechanisms involved in ligand induced neurite outgrowth of GFRα2a and GFRα2c receptor isoforms. A, GFRα2a mediated neurite outgrowth was blocked by PP2 (Src inhibitor), U0126 (MEK1/2 inhibitor) and U73122 (PLCγ inhibitor). Activation of ERK1/2 by GFRα2a was attenuated by PP2 and U0126. **B,** Neurite outgrowth in GFRα2c was prevented by PP2 but not by U0126. PP2 inhibited ERK1/2 activations, but the inhibition of ERK1/2 did not prevent neurite outgrowth, suggesting that an alternate pathway downstream of Src may be responsible for GFRα2c induced neurite outgrowth.

GDNF has previously been reported to enhance the expression of CRMP2 in neuroblastoma cells (Kodama *et al.*, 2004). However, it is unclear if specific GFRα1 and GFRα2 isoforms contribute to the observation. Using Neuro2A stably expressing GFRα2a or GFRα2c isoform, the up-regulation of *CRMP3* but not other CRMPs, by GDNF and NTN was unexpected. GFRα2a and GFRα2c have different kinetics in the regulation of *CRMP3* expression, suggesting a difference in the underlying mechanisms.

The tissue-selective expression of various CRMP family members in the adult nervous system suggest the possibility of distinct functions of CRMPs in addition to growth cone collapse (Schweitzer *et al.*, 2005; Wang and Strittmatter, 1996). Recently, CRMP-3 was shown to be a direct target of calpain and the cleavage product functioned as a positive injury signal to cause neuronal death after cerebral ischemia and excitotoxicity (Hou *et al.*, 2006). The result of the enhanced expressions of CRMP-3 in GFRα2a and GFRα2c cells when stimulated with ligand may contribute to the sculpturing of the growth cones and possibly controlling the life span of the differentiated cells, this is but a speculation awaiting exploration.

Cholinergic and GABAergic neurons are two major types of nerve cells found in the cereberal cortex. Neuro2A has been used as a neuronal model in the studies of cholinergic and GABAergic cell fate selection (Manabe *et al.*, 2005). Ligand activated GFRα2a and GFRα2c regulated GABAergic but not cholinergic markers. More specifically, the transcripts of the two GABAergic markers, *GAD1* and *GAD2*, were differentially regulated by the GFRα2 isoforms. *GAD1* and *GAD2* code for two isoforms of glutamate decarboxylase (GAD) proteins, GAD_{67} and GAD_{65}, respectively. While GAD_{67} is distributed throughout the cytoplasm and in the cell bodies, GAD_{65} is highly enriched in axon terminals and associated with synaptic vesicles (Kaufman *et al.*, 1991; Mackie *et al.*, 2003; Martin and Rimvall, 1993). Moreover, knocking out *GAD1* gene but not *GAD2* in mice produced a major decrease in whole brain GABA (Asada *et al.*, 1996; Asada *et al.*, 1997; Kaufman *et al.*, 1991; Mackie *et al.*, 2003; Martin and Rimvall, 1993), leading to proposal that GAD_{67} and GAD_{65} have distinct roles in the GABAergic neuron. In this study, GFRα2a and GFRα2c differently regulated the gene expression of the two GAD isoforms, suggesting possible roles of GFRα2a and GFRα2c isoforms in the

development of GABAergic neuron. It is interesting to note that GFRα2 isoforms are highly expressed in the human cortex (Chapter 4, Figure 4.3) and may thus be involved in the development of GABAergic nerve cells in the cortex.

The differences in neuritogenic mechanism of GFRα2a and GFRα2c further substantiate the concept that GFRα2 receptor isoforms have different biochemical activities and may play distinct physiological roles.

Chapter 8 Conclusion and future studies

8.1 Conclusion

In this thesis the biochemical and morphological functions of GFRα1 and GFRα2 isoforms were investigated. It was found that these isoforms share some common properties and yet have distinct biochemical and neuritogenic functions. While GDNF and NTN induced neurite outgrowth through GFRα2a and GFRα2c, ligand activated GFRα2b was found to inhibit neurite extension in a RhoA-dependent manner. Such neuritogenic inhibitory activities of GFRα2b were also observed in the alternatively spliced GFRα1 isoform, GFRα1b. Thus, depending on the presence of specific GFRα isoforms, GDNF and NTN may regulate pro- or anti-neuritogenic activities. The cross-talks of different GFLs (GDNF and NTN) with the same receptor complex (GFRα2, RET and NCAM) was found to result in the activation of different miRNAs. Thus, the observations reported herein suggest that the interaction of GFLs and the receptor complex is more intricate than initially anticipated.

The emerging view is that the combinatorial interactions of the spliced isoforms of GFRα, RET and NCAM complexes with the GFLs may contribute to the complexity and accounts for the myriad of biological responses observed.

8.2 Future studies

8.2.1 Mechanism of ligand activated anti-neuritogenic activities of GFRα2b

Although the activation and involvement of RhoA in GFRα2b inhibitory activity have been demonstrated, the downstream mechanism/s of GFRα2b inhibition of neuritogenesis remains unresolved. It has been shown that the activation of RhoA-ROCK pathway by LPA leads to phosphorylation and hence deactivation of cofilin, an adaptor protein involved in actin depolymerization. Such phosphorylation of

cofilin is mediated by LIM-kinase, which is activated by ROCK and is responsible for the growth cone collapse induced by neuritogenic inhibitory factors, such as LPA (Tojima and Ito, 2004). Although the rapid phosphorylation of cofilin by ligand activated GFRα2b has been shown (Fig. 5.10), a more detail analysis of the mechanism is required. The involvement of LIM-kinase in the phosphorylation of cofilin and the contribution to the neurite outgrowth inhibitory will be of interest. How the phosphorylation of cofilin affects the reorganization of cytoskeleton when GFRα2b is activated will provide further insight into the mechanism/s underlying neuritogenesis.

8.2.2 Hetero-oligomerization of isoforms

It is currently unknown if the spliced variants interact physically to form complexes. It has been suggested that the pairs of GFRα bind to dimerized GDNF ligands and subsequently recruit dimers of the Ret co-receptor to transducer intracellular signaling (Airaksinen and Saarma, 2002). The endogenous co-expressions of the three GFRα2 isoforms in the human cortex and in BE(2)-C cells suggests the possibility that GFRα2 isoforms may be able to form oligomers *in vivo*. It is unknown currently, whether GFRα isoforms prefer to form heterodimers or homodimers when more that one receptor isoforms are present. The use of epitope tags of individual receptor isoforms will enable the determination of the formation of homo/heterodimer in the same cell. Understanding the mechanism of oligomerization may facilitate the understanding of physical interactions of the spliced isoforms and shed light on physiological relevance.

8.2.3 Relative ratios of GFRα isoforms expression may affect functions

The co-expression of GFRα2 isoforms was performed using the bicistronic pIRES vector by expressing GFRα2b in the proximal 3' multiple cloning site (Chapters 4 - 6). It has been suggested that the translation may be less efficient in the 3' multiple cloning site compared to that of the 5' multiple cloning site of a bicistronic pIRES system (Hennecke *et al.*, 2001). Hence, it is possible that the protein expression of GFRα2b (expressed by 3' multiple cloning site) may be lower than that of GFRα2a or 2c, which were expressed by 5' multiple cloning site in the co-expression model used. Nevertheless, GFRα2b inhibited the neuritogenic activities of the other GFRα2 isoforms. As the IRES is not regulatable, the amount of transgene expressed cannot be controlled and does not allow a closer study on the relative expressions of the isoforms in the same cells. Currently, the laboratory has generated retroviral vectors which allow co-infections and the possibility of selecting predetermined levels of expression of multiple transgenes (Liu *et al.*, 2000). By differentially expressing at various levels, it should be interesting to examine the possibility that the relative ratios of GFRα isoforms may have diverse functions.

8.2.4 RET activations and RET isoforms

Alternative mRNA splicing of *RET* generates at least two isoforms named RET9 and RET51, with cytoplasmic domains containing 12 and 14 tyrosine residues respectively. These tyrosine residues are potential targets for trans-phosphorylation by the tyrosine kinase enzyme upon binding and activation to GFR-GFL complex. Four of the tyrosine residues have been identified as docking sites for various cytoplasmic

adaptor proteins, including Grb7/10 (Tyr905), PLCγ (Tyr1015), Shc/ENIGMA (Tyr1062) and Grb (Tyr1096), activating several messenger pathways including PLCγ, Jun-N-terminal kinase (JNK), phosphotidylinositol-3-kinase (PI3K) and Ras-MAPK kinase pathways (Mason, 2000; Murakumo *et al.*, 2006). The cadherin-like domains in the extra cellular region of RET is crucial for the binding of GFRα-GFL complex (Kjaer and Ibanez, 2003). However, the domains or regions on GFRα2 involved in binding to RET remain unclear. It is not known whether truncated GFRα isoforms may differ from full-length isoforms in recruiting and interacting with RET receptors. It is tempting to speculate that different GFRα isoforms may preferentially recruit specific RET isoforms and differentially phosphorylate one or more RET tyrosine residues to transduce distinct intracellular signaling. Further investigation of the interaction between specific GFRα and RET isoforms would be of future interest.

8.2.5 Method development for simultaneous expressions detection of GFRα receptor isoforms

As GFRα2b and GFRα1b exert antagonistic activity on other GFRα receptor isoforms, it is important to identify their expressions *in vivo* in order to understand their physiological functions. The quantitative real time PCR developed in the current study allows the specific detection of GFRα2 isoforms in total RNA isolated from tissues samples. It is currently unknown if these receptor isoforms are co-expressed in the same cell population. Detection and visualization of the co-expression of the GFRα2 isoforms in tissues or brain regions of interests will facilitate the understanding of the physiological relevance of the combinatorial actions of these GFRα2 isoforms. As GFRα2b and GFRα2c have no unique sequence as compared to GFRα2a, the conventional methods of *in situ* hybridization or immunochemistry

cannot differentiate the isoforms expressions from each other. The concept of designing probes at the specific cross-over exon boundary of GFRα2 isoforms (primers designed for quantitative real time PCR, Chapter 4) may be employed in developing specific probe for *in situ* hybridization. With the availability of narrow-band width fluorescent quantum dots, it will be possible to discriminate as many as 5 or more targets simultaneously (Tholouli *et al.*, 2006). Differential tagging of these isoforms-specific probes may then allow the simultaneous detection of expression of GFRα2 isoforms.

8.2.6 *In vivo* studies of GFRα splice isoforms

The current study revealed the complexity of interactions between GDNF ligands and GFRα splice isoforms, using *in vitro* model. Future studies should extend the understanding of GFRα splice isoforms using *in vivo* models. Using Neuro2A model, we have revealed the molecular and signaling mechanisms of GFRα splice isoforms in regulation of neuritogenic functions. For future studies, the use of primary neurons may better elucidate the phenotypical and neuritogenic funcations of GFRα splice isoforms. Lastly, the role(s) of GFRα splice isoforms in developing nervous systems and maintainances of neuronal functions needed to be further understood using specific transgenic or gene targeting mouse model. However, one must consider the potential cross overs and compensation effects between the family members.

Chapter 9 Materials and methods

A. Cells culture

Cells culture

Mice neuroblastoma, Neuro2A (ATCC No. CCL-131), and the human neuroblastoma, BE(2)-C (ATCC No. CRL-2268) were purchased from ATCC (Manassas, VA, USA). Cells were grown in 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) at 37°C in 5% CO_2, as previously described.

Generation of Neuro2A cells expressing GFRα1 or GFRα2 isoforms.

The murine neuroblastoma cell line Neuro2A which expresses endogenous RET and NCAM was stably transfected with either murine GFRα2a, GFRα2b, GFRα2c, GFRα1a, GFRα1b or vector control, pIRESneo (Clontech, Palo Alto, CA, USA) using Fugene-6 (Roche Lifescience, Germany) and selected with 0.8 mg/ml of G418 (Promega, Madison, WI, USA) over a period of two months. Primers used for measuring GFR isoforms, RET and NCAM expression are listed in Table 9.1, as previously described (Too, 2003; Yoong *et al.*, 2005).

For co-expression studies of GFRα2b with other GFR isoforms, GFRα2a, GFRα2c or GFRα1a were cloned into the proximal 5' multiple cloning site (MCS-A), while GFRα2b was cloned into distill 3' multiple cloning site (MCS-B) of the bicistronic pIRES vector (Clontech, Palo Alto, CA, USA). All studies were carried out with three independent clones.

For co-expression studies of GFRα1b and GFRα1a, GFRα1b was cloned into MCS-A, while GFRα1a was cloned into MCS-B of the bicistronic pIRES vector (Clontech, Palo Alto, CA USA). For control, cells with only GFRα1b expressed in MCS-A or cells with only GFRα1a expressed in MCS-B were generated.

Table 9.1. List of primers used for amplification of mouse GFRα2, GFRα1 isoforms, Ret, NCAM and GAPDH.

Gene	Name of primer (Forward/Reverse)	Primer sequences (Forward/reverse)	Size of amplicon (bp)
GFRα2a	mGFR2A+5F/ mGFR2-518r	GCCTCTTCTTCTTTTTAGACGAA/ TGTCGTTCAG GTTGCAGGCCT	486
GFRα2b	mGFR2B+5F/ mGFR2-518r	GCCTCTTCTTCTTTTTAGGTGAG/ TGTCGTTCAG GTTGCAGGCCT	171
GFRα2c	mGFR2C+5F/ mGFR2-518r	GCCTCTTCTTCTTTTTAGGGACA/ TGTCGTTCAG GTTGCAGGCCT	87
GFRα1a	mGFRa1a-421F/ mGFRa1a-550R	CATATCAGATGTTTTCCAGCA/ TGGTACAGGGGGTGATGTAGG	123
GFRα1b	mGFRa1b-409F/ mGFRa1a-550R	CAGTCCCGTTCATATCAGTGGA/ TGGTACAGGGGGTGATGTAGG	123
Ret	mRet51-2891F/ mRet51-2975R	TTCTGAAGACAGGCCACAGGA/ CACTG GCCTCTTGTCTGGCT	84
NCAM	mNCAM-N-F282/ mNCAM-N-R419	TGTCAAGTGGCAGGAGATGC/ GGCGTTGTAGATGGTGAGGGT	137
GAPDH	mhGAPDH-R599/ mGAPDH-F456	GATGGCATGGACTGTGGTCA/ GATGGGTGTGAACCACGAGAA	143

B. Ligands stimulations for kinases studies

Ligands stimulation of cells

BE(2)-C or Neuro2A cells were seeded in DMEM with 10% FBS for 24 hours, followed by serum depletion (0.5% FBS) for 16 hours. The cells were then treated with 50 ng/ml of recombinant human GDNF (Biosource, Camarillo, CA, USA) or recombinant human NTN (ProSpec-Tany TechnoGene Ltd, Israel) in serum free media for the designated length of time at 37 °C.

Immunoblotting.

Phosphorylation of MAPK (ERK1/2) or Akt was analyzed as follows. Cells were initially seeded in DMEM with 10% FBS for 24 hours and the serum was then depleted (0.5% FBS) for 16 hours. The cells were then treated with 50 ng/ml of

GDNF or NTN in serum free media for different time courses at 37 °C. For dose response studies, cells were stimulated with different concentrations of ligands for 10 minutes at 37 °C. Control treatment with 1 M Sorbitol (Sigma, Saint Louis, Missouri, USA) was carried out simultaneously. The supernatants were then removed and cells were washed once with phosphate-buffered saline (PBS) and subsequently lysed in 2% SDS. Protein concentrations were estimated using BCA assay (Pierce, Rockford, IL, USA). ERK1/2 or Akt phosphorylation was analyzed by Western blot using phospho-specific antibodies according to the manufacturer's instructions (Cell Signaling Technology, Danvers, MA, USA). Blots were stripped with Restore™ Western Stripping Buffer (Pierce, Rockford, IL, USA) and reprobed with pan antibodies to verify equal loading of protein. Western blots were detected using UltraSignal Chemiluminscent kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Chemiluminescence was imaged and analyzed by Quantity One version 4.0 (Bio-Rad, Hercules, USA).

For studies of kinetic and dose response of ligand induced ERK1/2 activations, dot blot analysis was performed using BIO Dot™ Apparatus (Bio-Rad, Hercules, USA). Five micrograms of protein were loaded per well in triplicates. Densities of blots were imaged and measured by Quantity One 4.0 (Bio-Rad, Hercules, USA).

To study the effects of kinases inhibitors, MEK inhibitor U0126 (Promega, Madison, WI, USA), Src inhibitor PP2 (Chemicon International, Temecula, CA, USA), or PLCγ inhibitor U73100 (Biosource, Camarillo, CA, USA) were added 20 minutes prior to the treatment of GDNF and NTN.

Binding of [^{125}I] GDNF to GFRα2 isoforms transfected Neuro2A cells.

[^{125}I] GDNF (~1000 mCi/mmol) was prepared using Amersham Bolton and hunter reagent (Amersham Biosciences, Buckinghamshire, England). Briefly, 10 μg of recombinant human GDNF (Prospec-Tany Technogene, Israel) were labeled with 1 mCi of Bolton and Hunter reagent for one hour at room temperature according to manufacturer's instructions. The reaction was terminated by adding 10 μl 0.1% BSA. Radiolabeled GDNF was then purified through a Sephadex G-10 column.

Binding studies were carried out as previously described (Jing *et al.*, 1997). Briefly, 0.1 millions cells were seeded per well on 24-well Costar tissue culture plates for two days before the assay. Prior to the experiment, cells were placed on ice for 15-20 minutes and then washed once with ice-cold DMEM buffer, 25 mM HEPES, pH 7.0. Cells were then incubated at 4˚C for three hours with 0.2 ml of binding buffer (DMEM buffer, 25 mM HEPES, 2 mg/ml bovine albumin serum and Roche Complete Inhibitor Cocktail, pH 7.0) containing 50 pM [^{125}I]GDNF and various concentrations of unlabeled GDNF. At the end of incubation, cells were washed three times with 0.3 ml of ice-cold washing buffer, and lysed in 0.1% SDS containing 1 M NaOH. The radioactivity in lysates was measured in the Cobra II Auto Gamma Counter (Perkin Elmer Packard).

C. Neurite outgrowth and differentiation experiments

Assessment of neurite outgrowth in GFRα transfected Neuro2A cells.

Twenty to fifty thousands cells per well were seeded on six well plates overnight, in DMEM supplemented with 10% FBS. Cells were then incubated with media containing 0.5% FBS, with or without 50 ng/ml of recombinant human GDNF (Biosource, Camarillo, CA, USA) or NTN (ProSpec-Tany TechnoGene Ltd, Israel).

Cells were incubated for three more days. All-trans retinoic acid (Sigma, Saint Louis, Missouri, USA) (5 μM) was used as a positive control for inducing neurite outgrowth. Cells bearing neurite twice the length of the cell bodies were scored. More than 600 cells from three different fields were counted per well. The significance differences between ligands stimulated and control samples were calculated using paired Students t-test. A value of $P<0.05$ was considered significant.

Immunocytochemistry and confocal microscopy.

Cells were seeded on chamber slides and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. After three washes with PBS, cells were permeabilized and blocked with serum (Dako, Glostrup, Denmark 1:10), 0.5% Triton-X100 in 1 X PBS for 30 minutes at room temperature. Cells were then incubated with primary antibodies (diluted 1:200, in 0.1% Triton-X100, 0.1% BSA, 1 X PBS) for 1 h at 37 ˚C and then washed three times in 1 X PBS. Secondary antibody (Alexa Fluor 488, Molecular Probes, USA) was then added at a dilution of 1:200 and incubated for one hour. The cells were then washed in 1 X PBS and mounted for visualization. Image acquisition was obtained using confocal microscope equipped with fluorescence detection (Zeiss, Thornwood, NY, USA).

F-actin (Phalloidin conjugated with TRITC) and high-molecular-weight neurofilament protein (NF-200) antibody, anti-HA (conjugated with TRITC) were purchased from Sigma (Sigma, Saint Louis, Missouri, USA); monoclonal beta-III Tubulin antibody was purchased from R&D System (Minneapolis, MN, USA).

Silencing of GFRα2b in BE(2)-C.

siRNA duplexes (Invitrogen, Carlsbad, CA, USA) were designed across the specific exon (exon 1 and 3) boundary of GFRα2b (as listed in Table 9.2). Sub-confluent cells (50-80%) were seeded on six well plates, in 10% FBS DMEM. Cells were transfected with siRNA duplexes (20pmol) using Transfectin (Bio-Rad, Hercules, USA) in 400µl of 0.5% FCS DMEM per well. Total RNA was isolated for six hours post-transfection, and gene expression was measured by real time PCR. For differentiation studies using BE(2)-C, six hours after silencing of GFRα2b, 2ml of differentiation medium containing retinoic acid (5µM), GDNF (50 ng/ml) or NTN (50 ng/ml) in 0.5% FBS DMEM was added to the media. Morphological differences were observed three days later.

Table 9.2 Designs of siRNA for human GFRα2b.

Name of siRNA design	Sense Sequence
siGFRα2b-15+5	TCTTCTTCTTTCTAG**GTGAG**
siGFRα2b-13+7	TCTTCTTCTTTCTAG**GTGAGGA**
siGFRα2b-10+10	TTCTTTCTAG**GTGAGGAGTT**
siGFRα2b-7+13	TTTCTAG**GTGAGGAGTTCTA**
siGFRα2b-5+15	TCTAG**GTGAGGAGTTCTACG**

Series of siRNA were designed across exon 1-3 of GFRα2b, underlined are sequences on exon 3 of GFRα2b.

Rho activation assay

Neuro2A cells were seeded in 10% FBS DMEM and incubated for 18-24 hours. Subsequently, the serum was reduced to 0.5% in DMEM and the cells were incubated for a further 18-24 hours. Cells were then treated with 10µM LPA (Sigma), GDNF (50 ng/ml) or NTN (50 ng/ml) in serum free DMEM for 10 minutes. Cells were lysed and used directly for the GTP-RhoA pull down assay according to the manufacturer's

instructions (Pierce, Rockford, IL, USA). RhoA inhibitor exoenzyme C3 transferase and ROCK inhibitor Y27632 were purchased from Calbiochem. Exoenzyme C3 transferase was transfected into cells using lipotransfecting agent, Transfectin (Biorad, Hercules USA), at 1 µl of Transfectin/ 1 µg of C3 transferase per well of 6-well plate. This was done four hours prior to the start of the experiment. Cells were then treated with RhoA inhibitor exoenzyme C3 transferase (1 µg/ml) or ROCK inhibitor Y27632 (10µM), with or without the presence of a differentiating medium. Lysophosphatidic acid (LPA, 10µM) (Sigma, Saint Louis, Missouri, USA) was used as a positive control for activities of Rho and ROCK inhibitor.

The plasmid of HA-tagged dominant negative RhoA (T19N) was a generous gift from Dr. Low Boon Chuan of Department of Biological Science, National University of Singapore. The dominant negative RhoA (RhoA-DN) was transfected to cells using Transfectin (Bio-Rad, Hercules, USA), 14-16 hours prior to the start of the experiment. Cells expressing RhoA-DN were then treated with differentiation media for three additional days, and were immunostained for beta-III Tubulin and monoclonal anti-HA TRITC conjugate (Sigma, Saint Louis, Missouri, USA), as described above.

Neuronal differentiation of mice embryonic stem cells

Two cell lines of mice embryonic stem cells (mESC) were used in this study, the AB2.2 and E14 cells. mESC was maintained on high-glucose DMEM (Gibco, CA USA) containing 15% FBS (Hyclone, Logan, UT, USA), 1x glutamine-pen-strep (Gibco, CA, USA), leukemia inhibitory factor (LIF) (1,000 U/ml) (Chemicon International, Temecula, CA, USA), and 143 µM β-mercaptoethanol (Merck, Germany), in a 5% CO_2 humidified atmosphere at 37 ˚C. Cells were passaged every

three days, with daily replacement of fresh media. Cells were cultured on flasks coated with 0.1% gelatin (Sigma, Saint Louis, Missouri, USA), for at least one hour at 37 ºC. Neuronal precursors were induced using retinoic acid and 4-4+ methods as previously described (Bain *et al.*, 1995; Bibel *et al.*, 2004). Briefly, embryoid bodies were induced by plating 3 $X10^6$ mESC cells onto non-adherent 10 cm bacterial dishes in EB medium (mESC medium without LIF and only 10% FBS) and incubated for eight days. Medium was changed every two days, and 5 μM retinoic acid was added after four days of embryoid bodies induction. Total RNA and reverse transcription were carried out as described above. Gene expressions of GFRα1 and Ret isoforms were measured by quantitative real time PCR, using specific primers as listed in Table 9.1.

D. Quantitative real time PCR

Isolation of RNA and reverse transcription

Total RNA from cells were prepared and the integrity of the samples were validated by denaturing agarose gel electrophoresis as described previously (Too and Maggio, 1995). One to five micrograms of total RNA were reverse-transcribed using 400U of ImpromII and 0.5 μg random hexamer (Promega, Madison, WI, USA) for 60 minutes at 42 °C according to the manufacturer's instructions. The reaction was terminated by heating at 70 °C for 5 minutes and used directly for quantitative real time PCR. Six independent preparations of cDNA were used for the study. All measurements were carried out in triplicates.

Quantitative real time PCR

Quantitative real time PCR was performed on the iCycler iQ (Bio-Rad, Hercules, USA) using Sybr Green I. The threshold cycles (Ct) were calculated automatically using the Optical interface v3.0B. Real time PCR was performed after an initial denaturation for 3 minutes at 95 ^{o}C followed by 40 to 50 cycles of 60 seconds denaturation at 95 ^{o}C, 30 seconds annealing at 60 ^{o}C and 60 seconds extension at 72 ^{o}C. Fluorescent detection was carried out at the annealing phase. The reaction was carried out in a total volume of 50 µl in 1X XtensaMix-SG^{TM} (BioWORKS, Singapore), containing 2.5 mM $MgCl_2$, 10 pmol of primers and 1.25 U Platinum DNA polymerase (Invitrogen Carlsbad, CA, USA). Melt curve analyses and agarose gel electrophoresis were carried out at the end of PCR to verify the identity of the products.

All real time PCR quantifications were carried out simultaneously with linearized plasmid standards and a non template control. The gene expression levels were interpolated from standard curves and normalized to the expressions of GAPDH in the same samples.

Quantification of miRNAs precursors

Total RNA was extracted from BE(2)-C cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) with linear acrylamide (20 µg/ml; Ambion Inc, Austin, TX, USA). The concentration of total RNA and the integrity of the RNA were quantified by absorbance (260 nm) and by 1% denaturing agarose gel electrophoresis. Further verifications were carried out using Experion^{TM} RNA StdSens analysis kit (Bio-Rad, Hercules, USA). Total RNA samples were treated with RNase-free DNase I (Promega, Madison, WI, USA) and inactivated at 80 $^{\circ}$C for 5 minutes, according to

manufacturer's recommendations. The DNAse I treated RNA samples were initially heated at 80°C in the presence of 15 pmol of pre-validated gene specific reverse primers (listed in Table 9.3) (Schmittgen *et al.*, 2004) for 5 minutes, snapped chilled on ice and reverse transcribed [1× buffer, dNTPs (10 mM), dithiothreitol (DTT), RNase inhibitor, Thermoscript (15 U)] as specified by the manufacturer (Invitrogen, Carlsbad, CA, USA). The reverse transcription was carried out at 60 °C for 45 minutes and terminated by a further incubation at 85 °C for 5 minutes. The samples were then used for quantitative real time PCR as described above using gene specific primers (listed in Table 9.3) (Schmittgen *et al.*, 2004). The real time PCR primers used here amplify both the short hair-pin structures of the miRNA precursors, primary (pri-miRNAs) and pre-miRNAs. The fold changes in the target miRNA, normalized to U6 RNA and relative to the expression in control sample, were calculated for each sample using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{miRNA} - Ct_{U6RNA})_{stimulated} - (Ct_{miRNA} - Ct_{u6RNA})_{control}$.

Table 9.3 List of primers used for amplification and measurement of human pri-miRNA.

miRNA	Name of primer (Forward/Reverse)	Primer sequences (Forward/reverse)
U6	U6F/ U6R	CTCGCTTCGGCAGCACA/ AACGCTTCACGAATTTGCGT
let-7d	let-7dF/ let-7dR	AACGCTTCACGAATTTGCGT/ AAGGCAGCAGGTCGTATAGT
miR-15	miR-15aF/ miR-15aR	GTAGCAGCACATAATGGTTTGTG/ GCAGCACAATATGGCCTG
miR-16	miR-16F/ miR-16R	GCAGCACGTAAATATTGGCGT/ CAGCAGCACAGTTAAATACTGGAG
miR-18	miR-18F/ miR-18R	TAAGGTGCATCTAGTGCAGATAG GAAGGAGCACTTAGGGCAGT
miR-20	miR-20F/ miR-20R	GCACTAAAGTGCTTATAGTGCAG/ GTACTTTAAGTGCTCATAATGCA
miR-21	miR-21F/ miR-21R	GCTTATCAGACTGATGTTGACTG/ CAGCCCATCGACTGGTG

miR-24-2	miR-24-2F/ miR-24-2R	CTCCCGTGCCTACTGAGCT/ CCCTGTTCCTGCTGAACTGAG
miR-28	miR-28F/ miR-28R	GGAGCTCACAGTCTATTAGTTACC / CCTCCAGGAGCTCACAATCT
miR-29	miR-29F/ miR-29R	ATGACTGATTTCTTTTGGTG/ ATAACCGATTTCAGATGGTG
miR-30a	miR-30aF/ miR-30aR	GTAAACATCCTCGACTGGAAGCT/ GCTGCAAACATCCGACTGAA
miR-30d	miR-30dF/ miR-30dR	GTTGTTGTAAACATCCCCGAC/ GCAGCAAACATCTGACTGAAAG
miR-33	miR-33F/ miR-33R	TGTGGTGCATTGTAGTTGCA/ CTGTGATGCACTGTGGAAAC
miR-92-1	miR-92-1F/ miR-92-1R	TCTACACAGGTTGGGATCGG/ CGGGACAAGTGCAATACCATA
miR-93-1	miR-93-1F/ miR-93-1R	AAGTGCTGTTCGTGCAGG/ CTCGGGAAGTGCTAGCTCA
miR-101	miR-101F/ miR-101R	GCCCTGGCTCAGTTATCACA/ GCCATCCTTCAGTTATCACAGTA
miR-105-1	miR-105-1F/ miR-105-1R	CAAATGCTCAGACTCCTGTGGT/ GCACATGCTCAAACATCCGT
miR-107	miR-107F/ miR-107R	CAGCTTCTTTACAGTGTTGCCT/ GATAGCCCTGTACAATGCTGC
miR-124a-2	miR-124a-2F/ miR-124a-2R	TCCGTGTTCACAGCGGAC/ CATTCACCGCGTGCCTTA
miR-147	miR-147F/ miR-147R	CTAAAGACAACATTTCTGCACAC/ ATCTAGCAGAAGCATTTCCAC
miR-216	miR-216F/ miR-216R	TGGCTTAATCTCAGCTGGCA/ TGAGGGCTAGGAAATTGCTCT
miR-219	miR-219F/ miR-219R	TCCTGATTGTCCAAACGCAA/ GGGACGTCCAGACTCAACTCTC
miR-22	miR-220F/ miR-220R	CCACACCGTATCTGACACTTT/ CAGACCGCATCATGAACAC
miR-224	miR-224F/ miR-224R	GGCTTTCAAGTCACTAGTGGTTC/ CTTTGTAGTCACTAGGGCACCA

Plasmids constructions

To prepare the plasmid standards for quantitative real time PCR, open reading frame of human GFRα2 isoforms and GAPDH were subcloned into p-GEMT (Promega, Madison, WI, USA). For early response genes and transcriptional factors, partial sequences were subcloned into p-GEMT. XbaI or XmnI (Promega, Madison, WI,

USA) was used to linearize plasmids to be used as templates for real time PCR amplifications.

Quantitative real time PCR of human GFRα receptor, RET and NCAM in BE(2)-C

Specific primers were designed to amplify the 3' GPI (glycosylphosphatidylinositol) sequences of GFRα1 or GFRα2, and did not distinguish between various alternatively spliced isoforms. Primers used for amplifying GPI region of GFRα1 and GFRα2, the common tyrosine kinase domain of RET and the N-terminal domain of NCAM, are listed in Table 9.4. All real time PCR quantification was carried out as described above, simultaneously with linearized plasmid standards and no template controls. The concentrations of GFRα, RET and NCAM in BE(2)-C cells were interpolated from standard curves and normalized to the expressions of GAPDH.

Table 9.4 List of primers used for amplification of human GFRα2, GFRα1, Ret, NCAM and GAPDH.

Gene	Name of primer (Forward/Reverse)	Primer sequences (Forward/reverse)
GFRα2	huGFRα2-1F/ huGFRα2-526R	ATGATCTTGGCAAACGTCTT/ TCTTGCAGTTGTCATTCAGGT
GFRα1	HuGFR1-GPI 1220F/ HuGFR1-GPI 1364R	TGTCGGGCAATACACACCTC/ AGAGCGGTTACCACCAGGA
RET	RET-NF/ RET-NR	TTCATCGGGACTTGGCAGC/ ACCATACATCACTTTGCGTG
NCAM	hNCAMC F341/ hNCAMC R547	CAGCAGCGGATCTCAGTGGT/ CATCACACACAATCACGGCA
GAPDH	huGAPDH-435F/ huGAPDH-597R	GATCATCAGCAATGCCTCCT/ GCCATCACGCCACAGTTT

Sequence independent real-time PCR for human GFRα2 isoforms.

Real time PCR was performed as described above. Specific exon overlapping forward primer used for amplification of human GFRα2a was designated as "2a 15+9F", for human GFRα2b as "2b 17+7F", and for human GFRα2c as "2c 18+5F". A common reverse primer, designated as "553R", was used for all three isoforms (listed in Table 9.5). All real time PCR quantification was carried out simultaneously with linearized plasmid standards and a non template control. Gene expression levels were interpolated from standard curves and normalized to the expressions of GAPDH in the same samples. Differences in the expression levels of GFRα2 isoforms were analyzed using paired Student's *t*-test with a level of significance of $P<0.05$.

Table 9.5 List of primers used for amplification of human GFRα2 isoforms and GAPDH.

Gene	Name of primer (Forward/Reverse)	Primer sequences (Forward/reverse)	Size of amplicon (bp)
GFRα2a	2a 15+9F/ 553R	TCTTCTTCTTTCTAGACGAGACCC / GCAGATGGAGATGTAGGAGGAG	545
GFRα2b	2b 17+7F/ 553R	CCTCTTCTTCTTTCTAGGTGAGGA / GCAGATGGAGATGTAGGAGGAG	233
GFRα2c	2c+5F/ 553R	GCCTCTTCTTCTTTCTAGGGACA/ GCAGATGGAGATGTAGGAGGAG	172
GAPDH	huGAPDH-435F/ huGAPDH-597R	GATCATCAGCAATGCCTCCT/ GCCATCACGCCACAGTTT	162

Measurement of early response genes and neuronal markers regulated by GDNF and NTN.

Cells were seeded in DMEM with 10% FBS for 24 hours, followed by serum depletion (0.5% FBS) for 16 hours. The cells were then treated with GDNF (50 ng/ml) or NTN (50 ng/ml) in serum free media for varying periods of time at 37 °C. Total RNA was then isolated and reverse transcribed as described above. The gene expression levels were then quantified by real time PCR using gene specific primers designed for early response gene, (listed in Table 9.6) or neuronal marker genes (listed in Table 9.7). Expression of target genes and GAPDH were interpolated from standard curves. Fold changes of target genes were calculated as changes in normalized gene expressions of stimulated samples as compared to control.

Table 9.6 List of primers used for amplification of mouse early response genes.

Gene	Name of primer (Forward/Reverse)	Primer sequences (Forward/reverse)	Size of amplicon (bp)
egr-1	EGR-1-328F/ EGR-1-459R	GAGAAGGCGATGGTGGAGACGA/ GCTGAAAAGGGGTTCAGGCCA	131
egr-2	EGR-2-1F/ EGR-2-179R	ATGAACGGAGTGGCGGGAGAT/ TCTGGATAGCAGCTGGCACCAG	179
c-fos	mcfos(B)651F/ mcfos(B)901R	TGTGGCCTCCCTGGATTT/ CTGCATAGAAGGAACCGGAC	250
fosB	mFosB(A)1926F/ mFosB(A)2107R	CAGGGTCAACATCCGCTAA/ GGAAGTGTACGAAGGGCTAACA	181

Table 9.7 List of primers used for measurement of mouse neuronal markers.

Gene	Name of primer (Forward/Reverse)	Primer sequences (Forward/reverse)	Size of amplicon (bp)
CRMP3	hmrCRMP3-265F/ hmrCRMP3-502R	TTCTGTCAGGGCACCAAG/ TGAAGACCAGGAAGGAGTTCAC	237
GAD1	mhGAD1-382F/ mhGAD1-787R	CGCAAGACATTTGATCGCT/ AGCAGCCATGATGCTGTACA	405
GAD2	mhGAD2-556F/ mhGAD2-870R	TTGGATATGGTTGGATTAGCAG/ TGCAGCTCCCTTCTTGAGAG	314
ChAT	hmChAT-148F/ hmChAT-631R	GAGCAGTTCAGGAAGAGCCA/ AGACGGCGGAAATTAATGAC	483

Chapter 10 References

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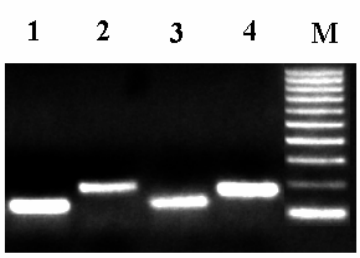
Chapter 11 Appendices

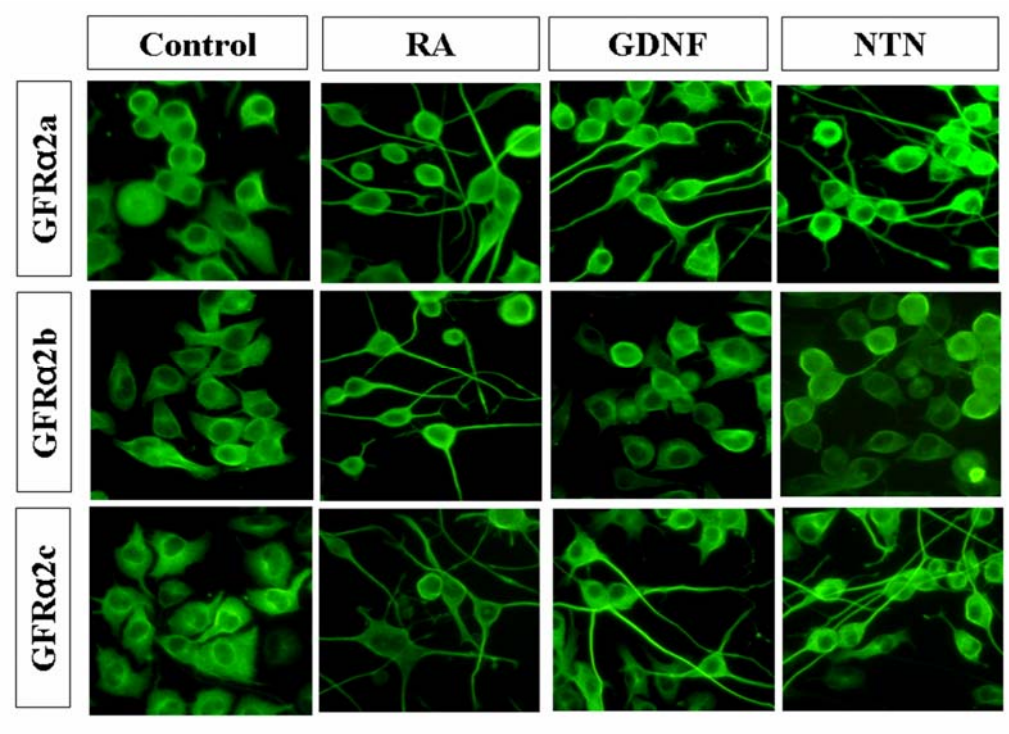
Supplementary figures

Appendix I:



Products of PCR amplification of GFRα1 (lane 1), GFRα2 (lane 2), Ret (lane 3), and NCAM (lane 4) from adult human brain. Primers were designed to recognize common C-terminal for GFRα1, GFRα2 or Ret, or to common N-terminal for NCAM (listed in Table 9.4), which were common to all isoforms. 100 bp marker (increment of 100bp for each band) was shown.

Appendix II



Immunocytochemistry of cytoskeletal component in ligand treated Neuro2A cells expressing GFRα2 isoforms. Figures show immunocytochemistry of beta III tubulin (green) in Neuro2A cells stably expressing GFRα2 isoforms, treated three days with retinoic acid (RA) (5 μM), GDNF or NTN (50 ng/ml). Magnification x200.

List of publications

1. Li Foong Yoong, Zhong Ni Peng, Guoqiang Wan, Heng-Phon Too. Tissue expression of alternatively spliced GFRa1, NCAM and RET isoforms and the distinct functional consequence of ligand-induced activation of GFRa1 isoforms, (**Molecular Brain Research** 2005, 139:1, 1-12).

2. Li Foong Yoong, Guoqiang Wan, Heng-Phon Too. Glial cell-line derived neurotrophic factor and Neurturin regulate the expressions of distinct miRNA precursors through the activation of GFRα2. (**Journal of Neurochemistry**, 2006, 98, 1149–1158)

3. Li Foong Yoong and Heng-Phon Too. Glial Cell Line-Derived Neurotrophic Factor and Neurturin Inhibit Neurite Outgrowth and Activate RhoA through GFRα2b, an Alternatively Spliced Isoform of GFRα2 (**Journal of Neuroscience**, 2007, 27:21, 5603-5614)

4. Li Foong Yoong, Zhong Ni Peng, Guoqiang Wan, Heng-Phon Too. GFRα1b inhibits GFRα1a neuritogenic activities in GDNF and NTN signaling. (Manuscript in preparation for **Journal of Neurochemistry**).

5. Leong DT, Gupta A, Bai HF, Wan Q, Yoong LF, Too HP, Chew FT, Hutmacher DW. Absolute quantification of gene expression in biomaterials research using real time PCR. (**Biomaterials**, 2007, 28, 203–210).

Abstracts communicated

1. YOONG Li Foong, PENG Zhong Ni, TOO Heng-Phon, Differential Expressions of GFRα1 and Ret Alternatively Spliced Isoforms In Murine Embryonic and Adult Tissues, 7th National University of Singapore- National University Hospital Annual Scientific Meeting, Singapore, 2003
2. YOONG Li Foong, Wan Guo Qiang, Peng Zhong Ni, Too Heng Phon. Expression and transcriptional profiles of GFR-Alpha1 alternatively spliced isoforms, 2nd International Congress of Neuroscience, Singapore, 2004

3. YOONG Li Foong, Too Heng-Phon, Distinct signaling and functional differences between GDNF family receptor alpha-2 (GFRα2) isoforms , 20th Biennial Meeting of the International Society for Neurochemistry, Austria 2005.

4. YOONG Li Foong, Too Heng-Phon; Novel antineuritogenic activity of GDNF Family Receptor Alpha 2b (GFRα2b), 2006 ASBMB (American Society for Biochemistry and Molecular Biology) Annual Meeting, San Francisco 2006.

5. YOONG Li Foong, Too Heng-Phon; GDNF Family Receptor Alpha 2b (GFRα2b) is an inhibitory receptor splice variant that antagonize GFRα2a and GFRα2c induced neurites extension, 20th IUBMB Congress and 11th FAOBMB Congress, Japan 2006.

6. YOONG Li Foong, Too Heng-Phon; GDNF Family Receptor Alpha 2b (GFRα2b) is an inhibitory receptor splice variant that antagonize GFRα2a and GFRα2c induced neurites extension, 7th Bienniel Meeting of the Asian Pacific Society for Neurochemistry (APSN), Singapore 2006.

Invited seminars and presentations

1. The novel anti-neuritogenic function of alternatively spliced glial cell line-derived neurotrophic factor (GDNF) receptor isoforms, at Howard Florey Research Institute, Melbourne Australia, September 2006.

2. The novel anti-neuritogenic function of alternatively spliced glial cell line-derived neurotrophic factor (GDNF) receptor isoforms, at St. Jude Children's Research Hospital, Memphis USA, October 2006.

3. YOONG Li Foong; Studies of GDNF Family Receptor Alpha 2 (GFRα2) isoforms, Young Scientists & Students session, 8th Biennial Advanced School of Neurochemistry of ISN (International Society of Neurochemistry), Valladolid Mexico, Aug 2007. (awarded best oral presentation)

Reprints of publications

1. Li Foong Yoong, Zhong Ni Peng, Guoqiang Wan, Heng-Phon Too. Tissue expression of alternatively spliced GFRa1, NCAM and RET isoforms and the distinct functional consequence of ligand-induced activation of GFRa1 isoforms, (**Molecular Brain Research** 2005, 139:1, 1-12).

2. Li Foong Yoong, Guoqiang Wan, Heng-Phon Too. Glial cell-line derived neurotrophic factor and Neurturin regulate the expressions of distinct miRNA precursors through the activation of GFRα2. (**Journal of Neurochemistry**, 2006, 98, 1149–1158)

3. Li Foong Yoong and Heng-Phon Too. Glial Cell Line-Derived Neurotrophic Factor and Neurturin Inhibit Neurite Outgrowth and Activate RhoA through GFRα2b, an Alternatively Spliced Isoform of GFRα2 (**Journal of Neuroscience**, 2007, 27:21, 5603-5614)

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Research Report

Tissue expression of alternatively spliced GFRα1, NCAM and RET isoforms and the distinct functional consequence of ligand-induced activation of GFRα1 isoforms

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Abstract

Glial-cell-line-derived neurotrophic factor (GDNF) exerts its effect through a multi-component receptor system consisting of GFRα1, RET and NCAM. Two highly homologous alternatively spliced GFRα1 isoforms (GFRα1a and GFRα1b) have previously been identified. In this study, isoform specific real-time PCR assays were used to quantify the expression levels of GFRα1, RET and NCAM isoforms in murine embryonic and adult tissues. The expression levels of GFRα1b were found to be comparable to that of GFRα1a in peripheral tissues. However, GFRα1a was the predominant isoform expressed in the whole brain. The co-expressions of GFRα1 and the co-receptors were developmentally regulated and differentially expressed in some tissues. Microarray analyses of GFRα1 isoforms transfected cells stimulated with NTN showed distinct and non-overlapping gene profiles. These observations are consistent with the emerging view that the combinatorial interactions of the spliced isoforms of GFRα, RET and NCAM may contribute to the pleiotropic biological responses.

Theme: Development and regeneration
Topic: Neurotrophic factors: receptors and cellular mechanisms

Keywords: GFRa1; GDNF; NCAM; RET; Alternative spliced isoform

1. Introduction

Glial-cell-line-derived neurotrophic factor (GDNF) is a cysteine-knot protein and belongs to a structurally related family of neurotrophic factors which includes Neurturin (NTN), Artemin and Persephin [1]. These GDNF family ligands (GFLs) have been shown to support the growth, maintenance and differentiation of a wide variety of neuronal and extraneuronal systems. GFLs exert their effects through a multi-component receptor system consisting of the GFRα, RET and NCAM [38,42]. Presently, the genes encoding four GFRα-related proteins, GFRα1 (GDNF-Rα), GFRα2 (NTNR-α, TrnR2), GFRα3 and GFRα4 have been isolated [3,6,16,17,19,29,44,47,54]. The ligand selectivity of GFRα1 has been a subject of controversy. Some studies have shown that GDNF but not NTN binds specifically to GFRα1 [6,19]. However, these findings have not been confirmed by others, and more recent results showed that both GDNF and NTN bind and activate GFRα1 in transfected cells as well as midbrain dopaminergic, parasympathetic and submandibular gland neurons [17,34,51].

Alternative RNA splicing occurs in nearly all metazoan organisms as a means of producing functionally diverse polypeptides from a single gene. In a recent genome-wide microarray analyses, it is estimated that greater than 74% of the human multi-exon genes are alternatively spliced

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[18]. We and others have previously identified two highly homologous alternatively spliced GFRα1 isoforms [11,37,40], and these were named GDNFR-α (GFRα1a) and GDNFR-β (GFRα1b) [8,11,16]. Both isoforms are expressed at unknown quantities in various human and rodent tissues. In addition to the existence of spliced isoforms of GFRα1, multiple isoforms of GFRα2 [12,52] and GFRα4 [22,23,26] have also been reported. The alternatively spliced variants of RET [10,20,25] and NCAM [7,35,36] have been shown to have distinct biochemical and physiological functions. These observations are consistent with the emerging view that the combinatorial interactions of the spliced isoforms of GFRα RET and NCAM may contribute to the multi-component signaling system to produce the myriad of observed biological responses.

In an effort to understand the possible physiological contributions of the GFRα1 isoforms, it is essential to determine the quantitative expression levels of the receptor and the multi-component receptor system. Currently, the quantitative expression levels of these isoforms are unknown. This is due to the lack of a method to quantify these highly homologous sequences. Concurrent with this study, an attempt to quantify the relative ratio of GFRα1 isoforms in tissues has been reported [8]. In this study, we report the development of specific, sensitive and high throughput real-time PCR assays to quantify the expression levels of GFRα1, RET and NCAM isoforms in murine whole embryos and adult tissues. These isoforms were shown to be expressed at significant levels during development, more at later stages. In addition, we tested the hypothesis that the spliced GFRα1 isoforms have distinct functions by microarray analyses of NTN stimulated GFRα1 isoform transfected cells which showed no overlap of gene profiles, indicative of distinctive functional roles of these isoforms.

2. Materials and methods

2.1. Reverse transcription (RT) reaction

Total RNA was prepared as described previously [46]. Total RNA from different tissues and from whole embryo were isolated from murine (Balb c). Further purification to remove genomic DNA was carried out using Nucleospin RNA II (Macherey-Nagel, Germany) according to manufacturer's instructions. The integrity of total RNA was validated by denaturing agarose gel electrophoresis. Five micrograms of total RNA from each tissue was reverse-transcribed using 400 U of ImpromII and 0.5 μg random hexamer (Promega, Madison, WI, USA) for 60 min at 42 °C according to the manufacturer's instructions. The reaction was terminated by heating at 70 °C for 5 min and used directly for quantitative real-time PCR. Six independent batches of cDNA were used for the study. All measurements were carried out in triplicate. All experiments involving animals were carried out in accordance to the Institutional Animal Care and Use Committee of the National University of Singapore and were in accordance with the US Public Health Service Guide for the Care and Use of Laboratory Animals.

2.2. Sequence independent real-time PCR using SYBR Green I Plasmids construction

Unless stated otherwise, all templates were generated by reverse transcription (RT)-PCR using murine brain cDNA (Balb c), subcloned into pGEM-T (Promega), and all clones were verified by DNA sequencing. The complete open reading frame (ORF) of murine GFRα1a (GenBank accession number: AF014117) and GFRα1b (GenBank accession number: AF015172) were generated by PCR and subcloned into pIRESneo (Clontech). The complete ORF of GAPDH was generated using the GenBank sequence (accession number NM_008084).

Murine RET51 (GenBank accession number AF209436) and RET9 (GenBank accession number AY326397) templates were generated using the pairs of primers RET51−3251R (TCTTGGGAACCCAGTGCTAG)/RET51−3168F (ATGGATTGAAA ACAAACTCTATGGCAT) and RET9−3243R (GTTACAGAGAGTTGGGATG)/RET9−3168F (ATGGATTGAAAACAAACTCTATGGTA), respectively. RET tyrosine kinase domain (TK2) template was generated using the primers mRET51−2890F (TTCTGAAGACAGGCCACAGGA)/mRET51−2974R (CACTGGCCTCTTGTCTGGCT).

NCAM (N-terminal) template encoding the 5′ ORF sequences was generated with primers mNCAM-N-F282 (TGTCAAGTGGCAGGAGATGC)/mNCAM-N-R419 (GGCGTTGTAGATGGTGAGGGT). A common forward primer (mNCAM-14-F1, CTCGCCTCTGAGTGGAAACCG) was used to generate NCAM120, NCAM140 and NCAM180 with the respective specific reverse primers mNCAM-15-R77 (TCAGAGCAGAAGAAGAGTCACCGC), mNCAM-17-R+12 (CTACAGGGCCCTTTTCGGGTTCTGTC) and mNCAM-18-R+12 (CTACAGGGCCCTTTTCGGTCTTTGCT). *Xba*I (Promega) and *Xmn*I (Promega) were used to linearize pGEM-T and pIRESneo plasmids, respectively.

2.3. Sequence independent real-time PCR

Real-time PCR was performed on the iCycler iQ (Biorad, Hercules, USA) using Sybr Green I. The threshold cycles (Ct) were calculated using the Optical interface v3.0B. Real-time PCR was performed after an initial denaturation for 3 min at 95 °C followed by 40 to 60 cycles of 60 s denaturation at 95 °C, 30 s annealing at 60 °C and 60 s extension at 72 °C. Fluorescent detection was carried out at the annealing phase. The reaction was carried out in a total volume of 50 μl in 1× XtensaMix-SG™ (BioWORKS), containing 2.5 mM $MgCl_2$, 10 pmol of primers and 1.25 U Platinum DNA polymerase (Invitrogen). Melt curve analyses were carried out at the

end of PCR to verify the identity of the products. All real-time PCR quantification was carried out simultaneously with linearized plasmid standards and a negative water control. The concentrations of GFRα1, RET and NCAM in all of the tissues were interpolated from standard curves and then normalized to the expressions of GAPDH in the same tissues. Differences in the expression levels of isoforms were analyzed using paired Student's *t* test with a level of significance of $0.05 > P > 0.02$. The specificity of an assay (ΔCt/ε) is determined by the difference in the Ct values of the defined target and test templates normalized to the efficiency of target amplification (ε) as described previously [45].

NCAM120, NCAM140 and NCAM180 were amplified with the following pairs of primers mNCAM-14-F179 (GCCATCCCAGCCACCCTG)/mNCAM-15-R72 (GCAGAAGAAGAGTCACCGCAGAG), mNCAM-16-F120 (CTGCTCATGTGCATCGCTG)/mNCAM-17-R+8 (CTACAGGGCCCTTTTCGGGTTC) and mNCAM-18-F729 (CCAGTGGACAAGCCTCTGAGC)/NCAM-18-R+8 (CTACAGGGCCCTTTTCGGTCTT), respectively. For amplifying the common N-terminal sequences of NCAM, the same primers used to generate the template NCAM (N-terminal) were used (mNCAM-N-F282 and mNCAM-N-R419).

GFRα1a and GFRα1b were amplified using a common reverse primer mGFRα1a-550R (TGGTACAGGGGGTGATGTAGG) and the corresponding specific forward primers mGFRα1a-421F (CATATCAGATGTTTTCCAGCA) and mGFRα1b-409F (CAGTCCCGTTCATATCAGTGGA), respectively. For amplifying the common glycosylphosphatidylinositol (GPI) sequences shared by GFRα1a and GFRα1b, the pair of primers mGFRα1a-1240F (TGTCTTTCTGATAATGATTACGGA)/mGFRα1a-1407R (CTACGATGTTTCTGCCAATGATA) was used.

RET9 and RET51 were amplified with the same primers used to generate the plasmid templates as mentioned above. To amplify the common tyrosine kinase region (TK2) shared by RET9 and RET51, TK2 template was amplified with primers RET51–2890F (TTCTGAAGACAGGCCACAGGA) and RET51–2974R (CACTGGCCTCTTGTCTG GCT). GAPDH was amplified using primers mhGAPDH-R599 (GATGGCATGGACTG TGGTCA) and mGAPDH-F456 (GATGGGTGTGAACCACGAGAA).

2.4. Stable transfection of Neuro2A cells

Neuro2A cells were stably transfected with either GFRα1a, GFRα1b or pIRESneo (Clontech) vector control using Fugene-6 (Roche Lifescience) and selected with 0.8 mg/ml of G418 over a period of 6 months. The cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone USA) in a 37 °C humidified incubator with 5% CO_2.

2.5. Analysis of MAP Kinase (Erk/12) phosphorylation

Cells were seeded in DMEM with 10% FBS for 24 h followed by serum depletion (0.5% FBS) for 16 h. The cells were then treated with 50 ng/ml of GDNF, NTN, Artemin or Persephin (PreproTech, England) in serum free media for 15 min at 37 °C. Control treatment with 1 M sorbitol (Sigma) was carried out simultaneously. The supernatants were removed, and cells were washed once with phosphate-buffered saline (PBS) and subsequently lysed in 2% SDS. Protein concentrations were determined using BCA (Pierce, Rockland USA). MEK inhibitor U0126 (Promega) was added 15 min prior to the addition of either GDNF or NTN, and ERK1/2 phosphorylation was analyzed by Western blot using phospho-specific antibodies according to the manufacturer's instructions (Cell Signaling Technologies). Immunoblots of transfected cells with GFRα1 (R&D), NCAM (Chemicon) and RET (Santa Cruz) were carried out according to the manufacturer's instructions. Immunoblots were visualized with chemiluminescence detection using horseradish peroxidase (Pierce, Rockland, USA). Blots were stripped and reprobed with actin (Dako) or pan Erk1/2 (Cell Signaling Technology) antibodies to verify equal loading of protein.

2.6. Immunocytochemistry

Cells were seeded on cover slips and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After three washes with PBS, cells were permeabilized with 0.5% Triton-X100 in 1× PBS for 30 min at room temperature and blocked with normal rabbit serum (DAKO, 1:10) in 0.1% Triton X-100 in PBS for another 30 min. The cells were then incubated with antiphospho-Erk1/2 antibody (Sigma, MO, USA) in 0.1% Triton X-100, 0.1% BSA, 1× PBS for 1 h at 37 °C and then washed three times in 1× PBS. Secondary antibody (Alexa Fluor 488, Molecular Probes, USA) was then added at a dilution of 1:1000 and incubated for 1 h. The cells were then washed in 1× PBS and visualized. Image acquisition was obtained using microscope equipped with fluorescence and phase contrast microscopy (Zeiss). For quantification of phospho-ERK1/2 time course activations, intensities of fluorescence were measured with Fluostar Optima (BMG Labtech) at excitation of 485 nm and emission of 520 nm.

2.7. Microarray analyses and verification with real-time PCR

For microarray analyses, cells were seeded in DMEM with 10% FBS for 24 h, and the serum was reduced (0.5% FBS) for a further 16 h. The cells were treated with 50 ng/ml of NTN (PreproTech, England) in serum free media for 10 min at 37 °C. Two independent clones of GFRα1a and GFRα1b stably transfected cell lines were studied. Total

RNA was isolated as described above. Total RNA (2 μg) was reverse transcribed and biotinylated cRNA (20–30 μg) produced according to the manufacturer's instructions (CodeLink Expression Bioarray System, Amersham Bioscience). The biotinylated cRNA was fragmented and then hybridized to CodeLink Uniset Mouse I Bioarray (Amersham Bioscience) gene chips, washed and detected with Cy5–Streptavidin dye conjugate. Bioarrays were scanned with GenePix 3000B Array Scanner (Axon Instruments) and analyzed using CodeLink Expression Analysis Software Ver 2.0 (Amersham Bioscience). Initial studies with murine brain total RNA in three independent studies showed that the coefficient of variation of signal reproducibility was about 15%, and the sensitivity of detecting two-fold detectable change was about 98% confidence based on unpaired *t* test analyses. Differential expression was calculated as the increase between ligand stimulated and control samples. A gene was considered differentially expressed when the mean probe intensity is higher than background control, increase or decrease of at least two-fold and showed reproducible changes in the two independent clones studied. Some of these genes were then selected to be analyzed by comparative real-time PCR analyses [24]. Primer pairs were designed to yield a short amplicon (100–200 bp) and with Tm of 60 °C. Total RNA (5 μg) was converted to cDNA and analyzed by real-time PCR, as described above. All amplifications were run in triplicate, and each experiment was replicated at least twice. GAPDH was used as a reference gene for comparative analysis.

2.8. Data analyses

The fold change in the target gene, normalized to GAPDH and relative to the expression in control sample, was calculated for each sample using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH})_{stimulated} - (Ct_{target} - Ct_{GAPDH})_{control}$.

3. Results

3.1. Development of a highly specific and sensitive quantitative real-time PCR assay

The exon organizations of the murine spliced variants of GFRα1, RET and NCAM are shown in Fig. 1. GFRα1a contains an extra 15 nucleotides encoded by exon 5, which is absent in GFRα1b isoform. With the RET isoforms, RET9 contains a unique intronic sequence of 27 nucleotides (intron 9), and RET51 contains exon 20. NCAM140 does not possess any unique sequences. Exon 15 is present in NCAM120 but is absent both in NCAM140 and NCAM180. Based on the limited availability of unique

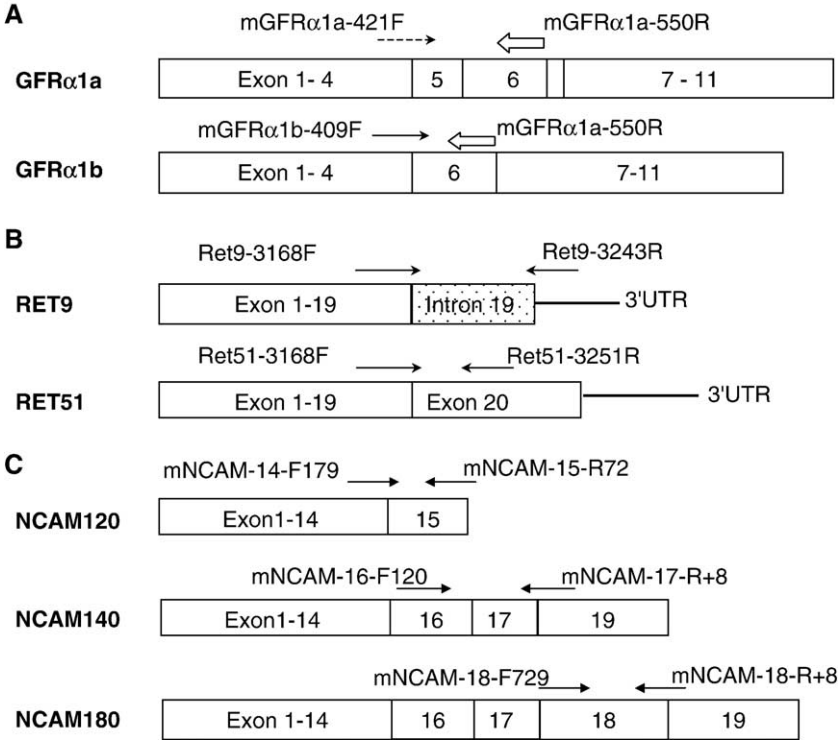

Fig. 1. Schematic diagram of the exon organizations of GFRα1[A], RET [B] and NCAM [C] alternatively spliced isoforms and the primers used for amplification. (A) A common reverse primer, mGFRα1a-550R (open arrows), was used with mGFRα1a-421F (dashed arrow) to amplify GFRα1a, and with mGFRα1b-409F (solid arrow) to amplify GFRα1b. (B) A specific primer spanning exon/intron 19 (Ret9–3168F) and a 3′ UTR primer (Ret9–3243R) were used to amplify RET9. RET51 was amplified with the exon spanning primer Ret51–3168F and Ret51–3251R. (C) NCAM isoforms were specifically amplified using exon overlapping primers. Forward primer for NCAM120 (mNCAM-14F179) was designed across exon 14/15; reverse primers for NCAM140 (mNCAM-17-R+8) and NCAM180 (mNCAM-18-R+8) were designed across exons 17/19 and 18/19, respectively.

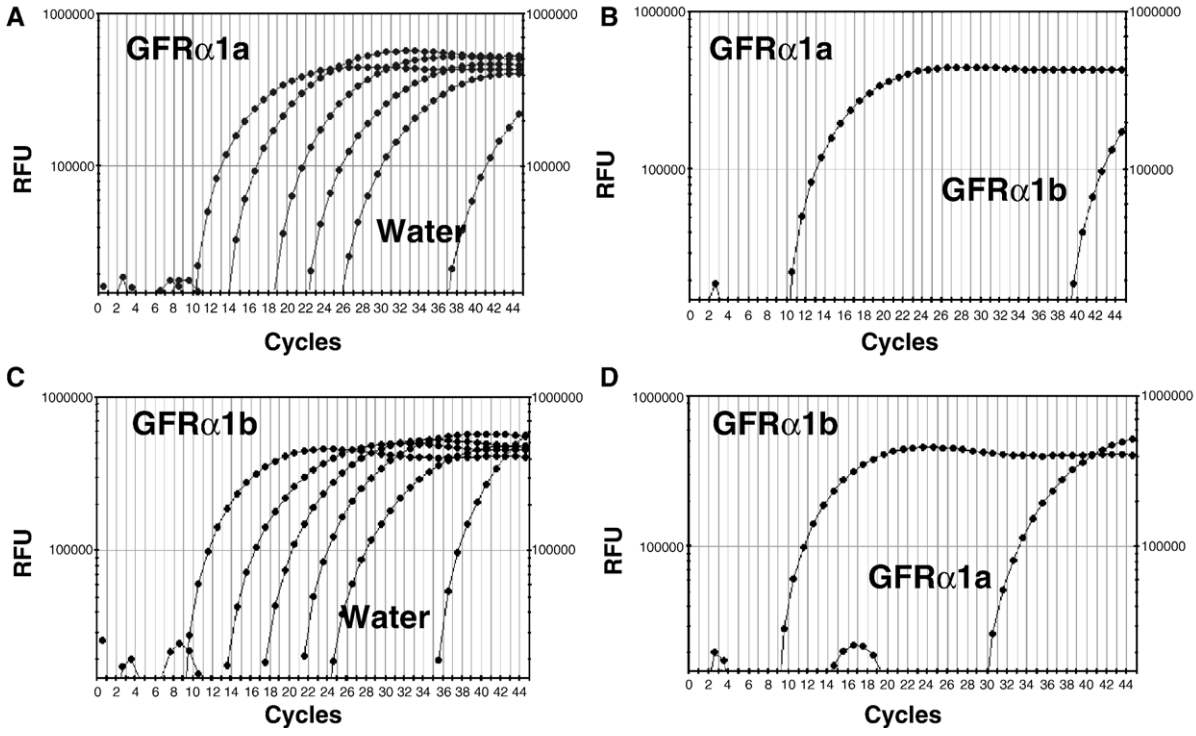

Fig. 2. Quantification of GFRα1 isoforms. Amplifications of five $\log_{10}$ dilutions of GFRα1a (A), GFRα1b (C), plasmid standards. Negative controls with no templates (Water) were carried out simultaneously. The slopes of the plots of Ct versus $-\log_{10}$ mol of GFRα1a and GFRα1b standards were 3.96 ± 0.38 (r^2 = 0.98) and 3.61 ± 0.21 (r^2 = 0.98), respectively. Equal quantities (6 attomol) of GFR isoforms were amplified separately using GFRα1a (B) and GFRα1b (D) exon specific primers. The experiments were repeated at least 5 separate times with similar results.

sequences in some of these isoforms, exon overlapping primers were designed and used for amplification (Fig. 1).

Initial studies were carried out to optimize the amplification conditions. A dwell time of 30 s for annealing, 60 s for denaturation at 95 °C and 60 s for extension at 72 °C were found to be optimal for the amplifications of the isoforms. There was no difference in the efficiencies of amplifying short amplicons of the exact size to the targets or those subcloned into plasmids. The use of hot-start polymerase increased the sensitivities of these assays significantly, allowing the detection of sub-zeptomoles of templates (Fig. 2). The slopes of the plots of Ct versus $-\log_{10}$ mol of all the standards were between 3.39 ± 0.30 (r^2 = 0.98) to 4.12 ± 0.44 (r^2 = 0.99). The specificities (σ) of the assays with hot-start polymerase were greater than 10^5 folds. For instance, amplifications of GFRα1b using GFRα1a exon overlapping primers were at least 10^5 fold less efficient than amplifying GFRα1a (Figs. 2B and D). Initial optimizations of the number of base pairs (bp) overlapping two adjacent exons were carried out (data not shown). The optimal number of bp overlaps was found to be dependent on the spliced variant examined. Hence, the use of 5 bp exon overlapping primer was the optimal length required to achieve high specificity of amplifications of the two GFRα1 isoforms. However, 8 bp exon overlapping primers were required to achieve high specificity of amplification for NCAM isoforms (Fig. 1C). Thus, the use of such exon overlapping primers under optimal conditions allowed the development of highly specific assays for the isoforms.

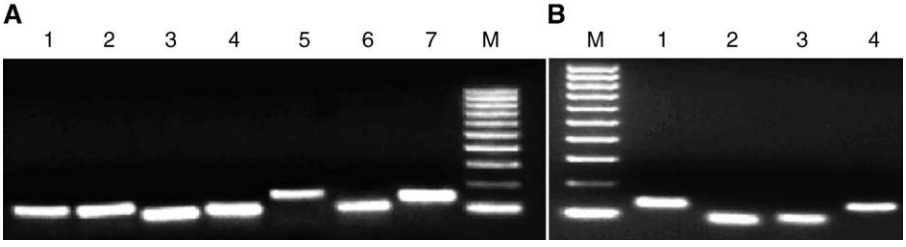

Fig. 3. Gel electrophoresis analysis of products after real-time PCR quantifications of adult murine brain transcripts. (A) Amplification products of GFRα1a (lane 1), GFRα1b (lane 2), RET9 (lane 3), RET51 (lane 4), GFRα1 glycosylphosphatidylinositol sequence (lane 5), RET encoding the tyrosine kinase region (lane 6) and GAPDH (lane 7). (B) Amplification products with primers designed to the N-terminal sequence of NCAM (lane 1), NCAM120 (lane 2), NCAM140 (lane 3) and NCAM180 (lane 4). Only bands corresponding to predicted sizes were observed. M, 100 bp DNA marker.

These assays were then used to quantify the expression levels of GFRα1, RET and NCAM isoforms transcripts in adult tissues and whole embryos at different stages of development. With all the assays, the efficiencies of amplifying isoforms from cDNA prepared from various tissues at different concentrations of RNA were identical to the respective standards used. Single products of the predicted sizes corresponding to the isoforms were observed by gel electrophoresis (Fig. 3). Melt curve analyses showed the predicted melting profiles. All amplified products were then subcloned and the sequences validated by DNA sequencing.

3.2. GFRα1b is expressed significantly in mouse tissues

The expression levels of GFRα1 in various murine adult tissues and whole embryo were shown in Fig. 4A. The sum of expression levels of GFRα1a and GFRα1b (Fig. 4A) quantified using specific isoform assays was similar to the levels determined using the assay designed to the common sequences (Fig. 4A). Both GFRα1a and GFRα1b were expressed at equivalent levels in all the tissues examined, except in the brain, where GFRα1a is the predominant isoform. Expressions of GFRα1 isoforms were very low at early stage of development (E7) but increased significantly in later stages. In the liver, both GFRα1 isoforms were expressed at similar levels but significantly higher than in other tissues. The expression of GAPDH in both brain and liver were equivalent when normalized to the amount of total RNA (data not shown).

3.3. RET9 is predominantly expressed in mouse tissues

Initial Northern blot analyses of murine tissues showed the existence of multiple hybridization bands (data not shown). As the probe used for Northern blot analyses do not discriminate between RET9 and RET51, we next employed specific real-time PCR assays to quantify these isoforms.

The highest expression of RET was found in the brain and testis (Fig. 4B). Similar to the results from Northern blot, the expressions of both RET isoforms in the kidney were found to be low. Although Northern blot analyses showed distinct hybridization signals in the liver, the expression of both isoforms was not detected either by the specific isoform assays (Fig. 4B) or the assays designed to the tyrosine kinase sequences (Fig. 4B). The expression levels of RET9 were significantly higher than that of RET51 in brain, heart, ileum, testis and the whole embryo samples. Both RET9 and 51 isoforms were expressed at low levels in the early (E7) as compared to the later stages of development. However, the third isoform of RET, RET 43, was not detected in any of the mouse tissues examined (data not shown). The sum of expression levels of RET9 and RET51 (Fig. 4B) quantified using specific isoform assays was similar to the levels determined using the assay designed to the common tyrosine kinase sequences (Fig. 4B).

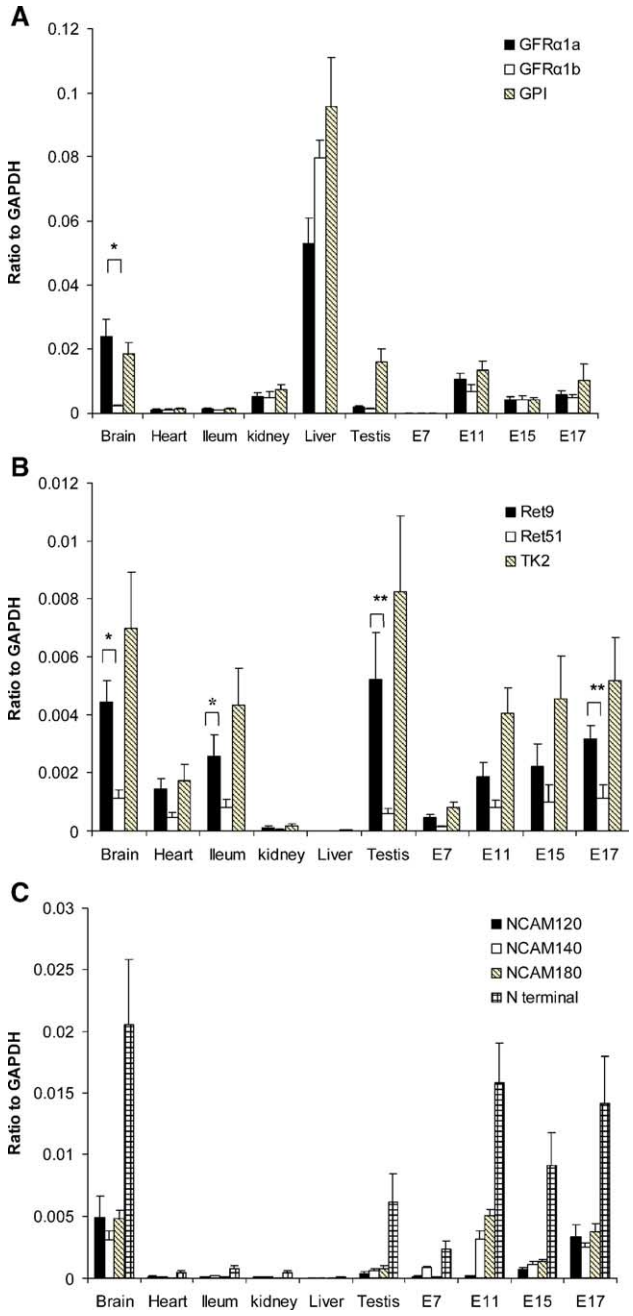

Fig. 4. Expression levels of GFRα1 and co-receptor isoforms in murine brain, heart, ileum, kidney, liver, testis and whole embryo of days 7, 11, 15 and 17. (A) Except for the brain, most tissues expressed comparable levels of GFRα1a and GFRα1b. (B) RET9 was significantly higher in expression than RET51 in most tissues. In the liver, the expression of RET9 and RET51 was below the detection limit of the assays. (C) Expression levels of NCAM120, NCAM140 and NCAM180. Expression of NCAM isoforms was undetectable in the liver. Expression levels of GFRα1 and co-receptors were determined using primers designed to the glycosylphosphatidylinositol sequence of GFRα1 (GPI), N-terminal sequence of NCAM (N-terminal) and tyrosine kinase sequence of RET (TK2). The expression levels were normalized to the levels of GAPDH in the same tissue. The results were expressed as mean ± SEM of at least 5 independent experiments. Significant differences in expression levels of GFRα1 (A) and RET (B) isoforms were calculated using paired Student's *t* test. A value of $P < 0.05$ were considered significant (*$P = 0.02$, **$P < 0.01$).

3.4. NCAM120, 140 and 180 are expressed at equivalent levels in the brain

All three NCAM transcripts were highly expressed in the brain but at low levels in most adult peripheral tissues (Fig. 4C). Differential expressions of NCAM isoforms were observed during development. The expression of NCAM120 increased at later stages of development (E15, E17), while NCAM140 and 180 were expressed at significant levels early in development (E11). The sum of expression levels of NCAM isoforms (Fig. 4C) quantified using specific isoform assays was similar to the levels determined using the assay designed to the common N-terminal sequences (Fig. 4C).

3.5. Neuro2A cells express NCAM and RET isoforms endogenously

To test the hypothesis that the spliced GFRα1 isoforms may have different functions, we used Neuro2A as a model as it expressed both NCAM and RET endogenously. At least 5 independent clones of stably transfected Neuro2A cells were analyzed for the expressions of GFRα1, NCAM and RET isoforms using real-time PCR and Western blot analyses (Fig. 5). Neuro2A cells express both RET (RET9 and RET51) and NCAM (NCAM120, 140 and 180) isoforms endogenously. The expression levels of RET and NCAM isoforms were similar in GFRα1a, GFRα1b or pIRES transfected Neuro2A cells. Initial studies using various commercially available antibodies to detect the expression of GFRα1 showed significant differences in the specificity and sensitivity of the Western blots. Specific GFRα1 isoforms expressions were only detected using antibody raised against the extracellular domain (R&D System) but not with antibodies directed at the C-terminal peptide GFRα1 (Santa Cruz and BD Pharmingen), both by immunocytochemistry and Western blot analysis (data not shown). Using the antibody directed at the extracellular domain, the expression levels of GFRα1a and GFRα1b were similar in transfected cells (Fig. 5B). Consistent with this observation, the expression levels of GFRα1 isoforms were similar at the transcript level (Fig. 5A).

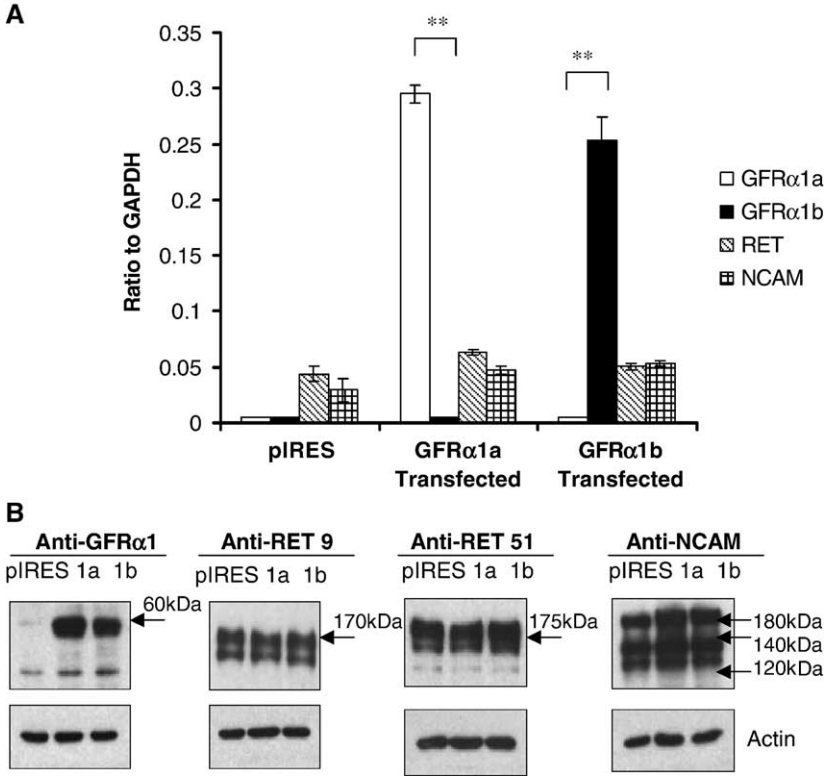

Fig. 5. Quantification of GFRα1, RET and NCAM expressions in stably transfected Neuro2A cells. (A) Real-time PCR analyses. GFRα1a and GFRα1b were expressed in cells transfected with the respective vectors and not in the pIRES vector control cells. Quantifications of the N-terminal sequences of NCAM (NCAM) and tyrosine kinase sequences of RET (RET) showed no significant change in the expression levels of endogenously expressed NCAM or RET in GFRα1 transfected cells. The results were expressed as mean ± SEM of at least 3 independent experiments. Significant differences in expression levels of GFRα1 isoforms were calculated using paired Student's t test. A value of $P < 0.05$ was considered significant (**$P < 0.01$). (B) Western blot analyses of GFRα1, RET and NCAM. GFRα1b (1b) was expressed at similar levels to GFRα1a (1a) when detected using anti-GFRα1 antibody. Vector control (pIRES) transfected cells showed no specific expression of GFRα1. NCAM isoforms (120, 140 and 180 kDa), RET9 (150 and 170 kDa) and RET51 (155 and 175 kDa) were expressed at similar levels in GFRα1a (1a), GFRα1b (1b) and vector control (pIRES) transfected cells. Blots were stripped and reprobed with antibody to actin to verify equal loading of samples.

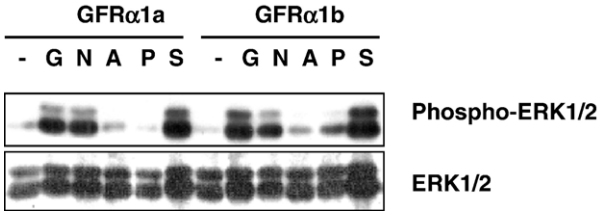

Fig. 6. Western blot analyses of ERK1/2 activation for GFRα1a and GFRα1b stably transfected Neuro2A cells stimulated with GFLS. (A) Cells were treated 10 min with 50 ng/ml of GDNF (G), NTN (N), Artemin (A), Persephin (P) or 1 M sorbitol (S). The blots were probed with antibody to phospho-ERK 1/2, stripped and reprobed with pan antibody to Erk1/2 (lower panel).

3.6. NTN and GDNF activate ERK1/2 in GFRα1 isoform transfected cells

When stimulated with either GDNF or NTN, both GFRα1a and GFRα1b induced the phosphorylation of ERK1/2 potently (Fig. 6). At the concentration (50 ng/ml) and time (10 min) studied, Artemin and Persephin did not significantly activate ERK1/2 when compared to sorbitol as a stimulus. GDNF or NTN stimulated ERK1/2 phosphorylation was rapid and transient (Figs. 7A and B). Phosphorylated ERK1/2 was localized to the nucleus rapidly and was inhibited by U0126 (Fig. 7A). ERK1/2 phosphorylation was specific to the MEK pathway as inhibitors of the p38 (SB 230580) and PI3 kinases (LY 294002) pathways did not inhibit ERK1/2 phosphorylation (data not shown). As a control, transfected cells with either GFRα1a or GFRα1b were stimulated with phorbol ester. No significant difference in the kinetics of ERK1/2 phosphorylation was observed (data not shown).

3.7. Profiles of ligand-induced gene expression in GFRα1 isoform transfected cells

To compare the gene expression profiles of NTN stimulated GFRα1 isoforms, microarray analyses of two

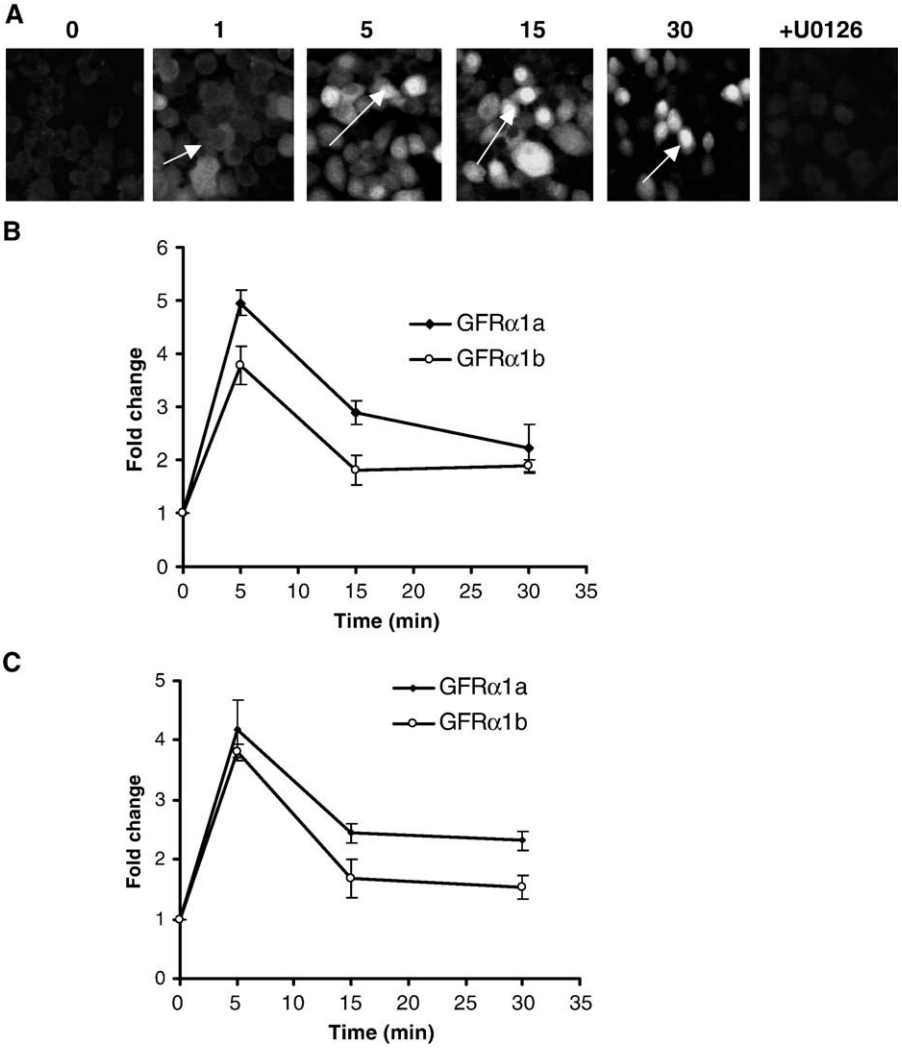

Fig. 7. Immunocytochemical staining of phosphorylated ERK1/2 in GFRα1a and GFRα1b transfected cells. (A) Cells were treated with 50 ng/ml of GDNF or NTN for the specified time. Nuclear localization of phosphorylated ERK1/2 was rapid (5 min), and the phosphorylation of ERK1/2 was inhibited by the MEK inhibitor U0126 (10 μM). (B, C) Quantification of phosphorylated ERK1/2 in GDNF (B) and NTN (C) stimulated GFRα1a and GFRα1b cells over a period of 30 min. The results were expressed as mean ± SD of triplicate readings. The experiment was repeated at least 3 separate times with similar results.

Table 1
NTN induced gene expression in GFRα1a and GFRα1b transfected cells at an early time point (10 min)

Accession number	Fold change	Gene annotations
GFRα1a		
NM023048	0.36	Ankyrin repeat and SOCS box-containing protein 4 (Asb4)
AK004405	0.54	Similar to KU70-Binding protein
AK005902	0.56	Hypothetical Trp–Asp repeat (WD-repeat) structure containing protein
AK009937	0.58	Hypothetical Cysteinyl-tRNA synthetase containing protein
AK010065	0.6	Unclassifiable
AK007332	0.68	Hypothetical 8.5 kDa Protein homolog
AK003917	1.33	Similar to BBP-like protein 2
AK016949	1.47	SWI/SNF related, matrix associated, actin dependent regulator of chromatin
AK016716	1.62	Similar to polymerase (RNA) III
GFRα1b		
NM022891	0.44	Ribosomal protein L23 (Rpl23)
NM023200	0.56	Protein phosphatase 1, regulatory (inhibitor) subunit 7 (Ppp1r7)
AF233643	0.65	Cytochrome P450 CYP4F13 mRNA
AK017628	0.7	Hypothetical mitochondrial energy transfer proteins (carrier protein)
NM080452	0.77	Mitochondrial ribosomal protein S2 (Mrps2)
AK009218	1.22	Nuclear protein skip
NM008206	1.34	Histocompatibility 2, O region alpha locus (H2-Oa)
AF360543	1.59	HNK-1 sulfotransferase
NM010375	3.16	Granzyme G (Gzmg)

The changes in gene expression were confirmed by real-time PCR using the comparative method ($2^{-\Delta\Delta Ct}$) and were statistically significant ($P < 0.05$). GenBank sequence accession number and gene annotations were assessed based on information supplied in the databases and from the literature.

independent clones of each isoform were performed. Focusing on the early changes in gene expression, a total of 10,000 genes were analyzed at 10 min of stimulation by NTN in duplicates. Approximately 0.4% and 0.2% of the total number of genes were stimulated in GFRα1a and GFRα1b, respectively. When stimulated by NTN, no overlap in the activated gene profiles between the two GFRα1 isoforms was observed. To validate the results, selected NTN stimulated samples were further analyzed for changes in expression levels by real-time PCR using comparative $2^{-\Delta\Delta Ct}$ method. It was found that the profile of changes in gene expression is consistent with the microarray analyses. No overlap in the gene expression profiles was observed between GFRα1a and GFRα1b cells when stimulated with NTN for 10 min, indicative of distinct functional differences between the two isoforms (Table 1).

4. Discussions

This is the first report using real-time PCR to quantify and compare the expression levels of GFRα1, RET and NCAM isoforms in tissues. To date, two distinct protein-coding alternatively spliced GFRα1 isoforms have been identified [11,37,40]. GFRα1b is identical to GFRα1a except that for the absence of 5 amino acids (140DVFQQ144), encoded by exon 5. Hence, GFRα1b do not have unique sequences for the design of amplification primers by PCR. Due to the lack of significant differences in nucleotide sequences, the detection and quantification of these isoforms often pose a challenge. We have previously shown that it is not possible to accurately quantify a homologous sequence using common amplification primers and specific fluorogenic probes [53]. By designing exon overlapping primers, it is however possible to discriminate and quantify highly homologous alternatively spliced sequences [45,53]. Concurrent with this study, an attempt to quantify the relative ratio of GFRα1 isoforms in tissues has been reported [8]. GFRα1a is expressed at significantly higher levels than GFRα1b in the central nervous system. Liver expresses high levels of both GFRα1a and GFRα1b, higher than that found in the brain. Consistent with this observation, we have previously detected high expression level of GFRα1 in the liver by Northern blot analyses, and the isoforms were subsequently isolated [11]. Both isoforms were detected only at later stages of development (E11–E17), unlike NCAM or RET expression [15].

The RET gene is alternatively spliced to yield two major isoforms, RET9 and RET51 [41]. RET is widely expressed in mammalian embryos and plays diverse roles in development [4,31,39,43]. During embryogenesis, the main sites of RET expression are in the nervous and excretory systems [30,50]. RET9 is critically important in kidney morphogenesis and enteric nervous system development, whereas RET51 appears dispensable [10]. It has been suggested that RET51 may be involved in differentiation events in later kidney organogenesis [20]. The two spliced variants recruit signal transducers in differentiation pathways differently and have dramatic biochemical differences on activation of GFRα1a [5,9,49]. In this study, significant level of RET was detected early in development (E7), similar to NCAM 140 but not NCAM 120/180 or GFRα1a. In most of the tissues examined, RET9 is the predominant isoform expressed. It is not known if the spliced GFRα1 isoforms may preferentially activate specific isoforms of RET.

GFRα are expressed more widely than RET in many regions of the nervous system in vivo [48,55], raising the possibility that RET-independent GFRα may act to capture and concentrate diffusible GFLs and subsequently presenting them in trans to RET-expressing cells to stimulate RET signaling [27,32]. Other evidence suggests that GFRα can mediate GFL signaling in a RET-independent manner through the neural cell adhesion molecule (NCAM) [33]. The physiological importance of trans-signaling and GFRα1-dependent NCAM signaling in the development of the nervous system or the kidney has recently been challenged [14]. However, the study could not rule out the

possibility of compensatory effects of other GFRαs. Interestingly, neither RET nor NCAM isoforms were detectable in the liver where high levels of GFRα1 isoforms were found, raising the possibility of the existence of yet to be identified co-receptor/s or signaling through RET/NCAM-independent pathway/s. Another possibility is that other alternatively spliced RET isoform not detected by the highly specific real-time PCR assay developed herein may exist. This is consistent with the Northern blot analyses with a probe encoding the tyrosine kinase sequence of RET showing bands smaller than 4 kb in certain tissues (liver, heart and testis). The identity of these bands is currently investigated.

GFRα1a appears to be structurally organized into distinct domains [13]. Domain 3 has been crystallized and has been used to model domain 2 [21]. Interestingly, the predicted domain 2 helices [2] showed the same positions of cysteine residues which are thought to form disulfide bridges as observed in the helices in domain 3. Both domains 2 and 3 are involved in the binding of GDNF. Domains 1 and 2 are thought to be linked by an extended loop (residues 114–144). The absence of the 5 amino acids (140DVFQQ144) in GFRα1b isoform may confer significant structural differences between the spliced isoforms which may have different functional consequences. To test the hypothesis that the spliced GFRα1 isoforms have distinct functions, we used DNA microarray to analyze NTN stimulation of GFRα1 isoforms in transfected cells. Both GFRα1 isoforms when stimulated by GDNF or NTN resulted in the rapid phosphorylation and nuclear localization of ERK1/2, specifically through the MEK pathway. An early time point was then chosen to examine the gene profile of NTN stimulation of GFRα1 isoforms. Stimulation with NTN resulted in specific gene changes which are not shared by the isoforms, indicative of distinctive functional roles. Consistent with this finding, a recent report showed biochemical differences in the activation of the spliced variants [8].

It is now believed that a substantial fraction of the human genes (~74%) produce alternatively spliced transcripts [18]. A significant proportion of these alternative-splicing events are thought to alter the resulting protein products, which may result in altered functions [28]. The differential expression and distinct functional response of spliced isoforms of GFRα1 are consistent with the emerging view that the combinatorial interactions of these spliced isoforms and the co-receptors may contribute to the multi-component signaling system to produce the myriad of observed biological responses.

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Glial cell-line derived neurotrophic factor and neurturin regulate the expressions of distinct miRNA precursors through the activation of GFRα2

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) are structurally related neurotrophic factors that have both been shown to prevent the degeneration of dopaminergic neurons *in vitro* and *in vivo*. NTN and GDNF are thought to bind with different affinities to the GDNF family receptor alpha-2 (GFRα2), and can activate the same multi-component receptor system consisting of GFRα2, receptor tyrosine kinase Ret (RET) and NCAM. MicroRNAs (miRNAs) are a class of short, non-coding RNAs that regulate gene expression through translational repression or RNA degradation. miRNAs have diverse functions, including regulating differentiation, proliferation and apoptosis in several organisms. It is currently unknown whether GDNF and NTN regulate the expression of miRNAs through activation of the same multi-component receptor system. Using quantitative real-time PCR, we measured the expression of some miRNA precursors in human BE(2)-C cells that express GFRα2 but not GFRα1. GDNF and NTN differentially regulate the expression of distinct miRNA precursors through the activation of mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2). This study showed that the expression of distinct miRNA precursors is differentially regulated by specific ligands through the activation of GFRα2.

Keywords: GDNF family receptor alpha-2, glial cell line-derived neurotrophic factor, microRNA, neurturin, receptor tyrosine kinase Ret.

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Glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), artemin and persephin are cysteine-knot proteins and are structurally related neurotrophic factors (Airaksinen and Saarma 2002). These GDNF family ligands (GFLs) have been shown to support the growth, maintenance and differentiation of a wide variety of neuronal and extraneuronal systems (Saarma and Sariola 1999; Airaksinen and Saarma 2002). GFLs exert their effects through a multi-component receptor system consisting of GDNF family receptor alpha (GFRα), receptor tyrosine kinase Ret and neural cell adhesion molecule (NCAM) (Airaksinen *et al*. 1999; Paratcha *et al*. 2003). Each GFL is known to bind preferentially to one GFR (*in vitro*), and the activation of the multi-component receptor system shows some degree of promiscuity in ligand specificity (Horger *et al*. 1998; Airaksinen *et al*. 1999; Cik *et al*. 2000; Wang *et al*. 2000; Scott and Ibanez 2001). Mice lacking GDNF, GFRα1 or RET share common phenotypes for kidney agenesis and the absence of many parasympathetic and enteric neurons (Enomoto *et al*. 1998, 2001; Cullen-McEwen *et al*. 2001). NTN or GFRα2 knock-out mice show similar deficits in parasympathetic and enteric innervations, but notable differences have been reported (Heuckeroth *et al*. 1999; Rosenthal 1999; Rossi *et al*. 1999). These phenotypic differences may be due to a different genetic background of

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Abbreviations used: Ct, threshold cycles; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotrophic factor; GFLs, GDNF family ligands; GFRα2, GDNF family receptor alpha-2; MAPK, mitogen-activated protein kinase; miRNA, microRNA; NCAM, neural cell adhesion molecule; NTN, neurturin.

the mice used or, more interestingly, may suggest the possibility of GDNF crosstalk through GFRα2 *in vivo*. GDNF has been used in clinical trials and the results were favorable in some reports (Gill *et al*. 2003; Slevin *et al*. 2005) but not in others (Nutt *et al*. 2003; Peggy 2005). Although crosstalk in development may be of limited significance (Airaksinen and Saarma 2002), it may be highly relevant when exogenous GFLs are applied *in vivo*.

MicroRNAs (miRNAs) are small, non-coding RNAs that serve as important post-transcriptional regulators of gene expression in metazoans. To date, a large number of miRNAs have been identified in diverse organisms, including vertebrates and plants (Dugas and Bartel 2004; Harfe 2005). The number of miRNA genes appeared to be greater than 1% of the predicted genes in humans (Lim *et al*. 2003; Bentwich *et al*. 2005). In many respects, miRNA genes resemble protein coding genes in that they may possess introns (Rodriguez *et al*. 2004) and are transcribed by RNA polymerase II (Lee *et al*. 2004). In addition, the transcripts from miRNA genes are capped, spliced and polyadenylated (Cai *et al*. 2004). Pre-miRNA sequences are predicted based on the folded structures and are derived from primary transcript, pri-miRNA (Bartel 2004). The mature miRNA (21–24 nucleotides) is located in the hairpin structure of pre-miRNA and is produced through at least two RNAse III-mediated steps (Lee *et al*. 2002; Du and Zamore 2005). It is now clear that miRNAs exert their functions by binding to complementary sequences on target mRNAs, inducing cleavage of mRNAs or repression of protein translation (Ke *et al*. 2003; Nelson *et al*. 2003; Bartel 2004; Pillai 2005). miRNAs have extensive regulatory roles, including involvement in development, cell proliferation, cell death and morphogenesis (Ambros 2003; Miska 2005; Pillai 2005).

It is currently unknown whether GDNF and NTN can regulate the expression of miRNAs in various cellular processes. In this study, the human BE(2)-C cell line, which express GFRα2 but not GFRα1, was used to examine the regulation of some miRNA precursors (pre-miRNAs and pri-miRNAs) by GDNF and NTN. Interestingly, the results showed that despite the promiscuity of ligand–receptor interaction, GDNF and NTN regulate the expression of distinct miRNA precursors through the activation of the mitogen-activated protein kinase (MAPK) [extracellular signal-regulated kinase (ERK1/2)] signaling pathways.

Materials and methods

Cell culture

Neuroblastoma BE(2)-C (CRL-2268) cells were purchased from ATCC (Manassas, VA, USA). Cells were grown in 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) at 37°C in 5% CO_2, as previously described (Kobori *et al*. 2004).

Isolation of RNA and reverse transcription

Total RNA from BE(2)-C cells was prepared and the integrity of the samples was validated by denaturing agarose gel electrophoresis, as described previously (Too and Maggio 1995). A 1–5 μg aliquot of total RNA was reverse-transcribed using 400 U ImpromII and 0.5 μg random hexamer (Promega, Madison, WI, USA) for 60 min at 42°C, according to the manufacturer's instructions. The reaction was terminated by heating at 70°C for 5 min and was used directly for quantitative real-time PCR. Six independent preparations of cDNA were used for the quantification of GFRα, NCAM and RET levels in BE(2)-C cells. All measurements were made in triplicate.

Quantitative real-time PCR of GFRα, NCAM and RET

Quantitative real-time PCR was performed on the iCycler iQ (Bio-Rad, Hercules, CA, USA) using Sybr Green I. The threshold cycles (Ct) were calculated automatically using OPTICAL INTERFACE v3.0B (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed after an initial denaturation for 3 min at 95°C, followed by 40–50 cycles of 60 s denaturation at 95°C, 30 s annealing at 60°C and 60 s extension at 72°C. Fluorescent detection was done at the annealing phase. The reaction was carried out, in a total volume of 50 μL, in 1× XtensaMix-SG (BioWORKS, Singapore) containing 2.5 mM $MgCl_2$, 10 pmol of primers and 1.25 U platinum DNA polymerase (Invitrogen Carlsbad, CA, USA). Melt-curve analyses and agarose gel electrophoresis were carried out at the end of PCR to verify the identity of the products. Specific primers were designed to amplify the 3′ GPI (glycosylphosphatidylinositol) sequences of GFRα1 or GFRα2, and do not distinguish between various alternatively-spliced isoforms. Primers used for amplifying GFRα1 were HuGFRα1-GPI 1220F (TGTCGGGCAATACACACCTC) and HuGFRα1-GPI 1364R (AGAGCGGTTACCACCAGGA). GFRα2 was amplified using HuGFRα-2a-1263F (CTTCACAGAGCTCAC-GACAAA) and HuGFRα-2a-1381R (TCAGCATCAGGACAGA-CAGC). A pair of primers (TTCATCGGGACTTG GCAGC/ ACCATACATCACTTTGCGTG) was designed to amplify the common tyrosine kinase domain of RET and does not distinguish between the isoforms (RET9 and RET51). The N-terminal domain of NCAM was amplified using primers hNCAMC-F341 (CAG-CAGCGGATCTCAGTGGT) and hNCAMC-R547 (CATCACA-CACAATCACGGCA). GAPDH transcript was amplified using primers huGAPDH-435F (GATCATCAGCAATGCCTCCT) and huGAPDH-597R (GCCATCACGCCACAGTTT). All real-time PCR quantifications were carried out simultaneously with linearized plasmid standards and no template controls. The concentrations of GFRα, RET and NCAM in BE(2)-C cells were interpolated from standard curves and normalized to the expressions of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Quantification of miRNAs precursors

Total RNA was extracted from BE(2)-C cells using Trizol reagent (Invitrogen) with linear acrylamide (20 μg/mL; Ambion Inc., Austin, TX, USA). The concentration of total RNA and the integrity of the RNA were quantified by absorbance (260 nm) and by 1% denaturing agarose gel electrophoresis. Further verifications were carried out using the Experion RNA StdSens analysis kit according to the manufacturer's instructions (Bio-Rad). Total RNA samples were treated with RNase-free DNase I (Promega) and inactivated at 80°C for 5 min, according to manufacturer's recommendation. The DNAse

I-treated RNA samples were initially heated at 80°C in the presence of 15 pmol of pre-validated, gene-specific reverse primers (Schmittgen *et al*. 2004) for 5 min, snap-chilled on ice and reverse transcribed [1 × buffer, dNTPs (10 mM), dithiothreitol (DTT), RNase inhibitor, Thermoscript (15 U)] as specified by the manufacturer (Invitrogen). The reverse transcription was carried out at 60°C for 45 min and terminated by a further incubation at 85°C for 5 min. The samples were then used for quantitative real-time PCR, as described above, using gene-specific primers (Schmittgen *et al*. 2004). The real-time PCR primers used here amplify both the short hairpin structures of the miRNA precursors, primary (pri-miRNAs) and pre-miRNAs. The fold changes in the target miRNA, normalized to U6 RNA and relative to the expression in control sample, was calculated for each sample using the equation: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{miRNA} - Ct_{U6RNA})_{stimulated} - (Ct_{miRNA} - Ct_{U6RNA})_{control}$.

Ligand stimulation of cells

BE(2)-C cells were seeded in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS for 24 h, followed by serum depletion (0.5% FBS) for 16 h. The cells were then treated with 50 ng/mL GDNF or NTN (PreproTech, London, UK) in serum-free medium for the designated length of time at 37°C. Control treatment with 1 M sorbitol (Sigma, St. Louis, MO, USA) was carried out simultaneously for the analyses of MAPK activation. To control for variability, two identical plates of cells were stimulated simultaneously. One of the two plates of cells was used for direct RNA isolation and the other was used for western blot analysis. For inhibitor studies, MEK1/2 inhibitor, U0126 (Promega), was added simultaneously with the ligands or, alternatively, cells were pre-treated with the inhibitor for 20 min prior to the addition of ligands.

Western blot analysis of MAPK (ERK1/2) phosphorylation

Ligand stimulations were terminated by removing the supernatant fluids, then the cells were washed once with phosphate-buffered saline (PBS). The cells were lysed in 2% sodium dodecyl sulfate (SDS) and protein concentrations were determined using bicinchoninic acid (BCA; Pierce, Rockford, IL, USA). ERK1/2 phosphorylation was analyzed by the SuperSignal western blotting kit (Pierce) using phospho-specific antibody (p-Thr202/204) according to the manufacturer's instructions (Cell Signaling Technology, Danvers, MA, USA). Blots were stripped and either re-probed with actin (Dako, Glostrup, Denmark) or pan ERK1/2 antibodies (Promega) to verify equal loading of proteins. For kinetic studies, total cell extracts (5 μg) were blotted onto Hybond-N (Amersham Biosciences, Little Chalfont, UK) using the BIO Dot Apparatus (Bio-Rad). Western blots were detected with ERK1/2 phospho-specific antibody (p-Thr202/204) using the UltraSignal Chemiluminscent kit (Pierce) according to the manufacturer's instructions. Chemiluminescence was imaged and analyzed by QUANTITY ONE version 4.0 (Bio-Rad).

Results

Neuroblastoma BE(2)-C cells express GFRα2, NCAM and RET

The quantitative real-time PCR assays designed for GFRα1 and GFRα2, NCAM and RET were highly sensitive (detection limit of 10 copies per reaction) and specific, showing only single products of the predicted sizes corresponding to the amplicons as observed by gel electrophoresis (data not shown). The amplification efficiencies of cDNA at different concentrations of RNA were greater than 95% and were identical to the respective standards used. Melt-curve analyses showed the predicted melting profiles, and all amplified products were validated by DNA sequencing. Using these assays, NCAM, RET and GFRα2, but not GFRα1, were detected in BE(2)-C cells (Fig. 1a). In BE(2)-C cells, the GFRα1 transcript level was below the detection limit of the assay and estimated to be less than $1 : 10^6$ when expressed as the ratio of GFRα1 to GAPDH. The absence of GFRα1 expression in BE(2)-C cells was not due to failure of detection by the real-time PCR assay as the same assay, when used to quantify the expression level of the transcript in adult human brain, showed significant levels of GFRα1 (a ratio of GFRα1 to GAPDH of 0.08 ± 0.002). Amplifications of cDNA samples prepared from BE(2)-C cells and whole adult

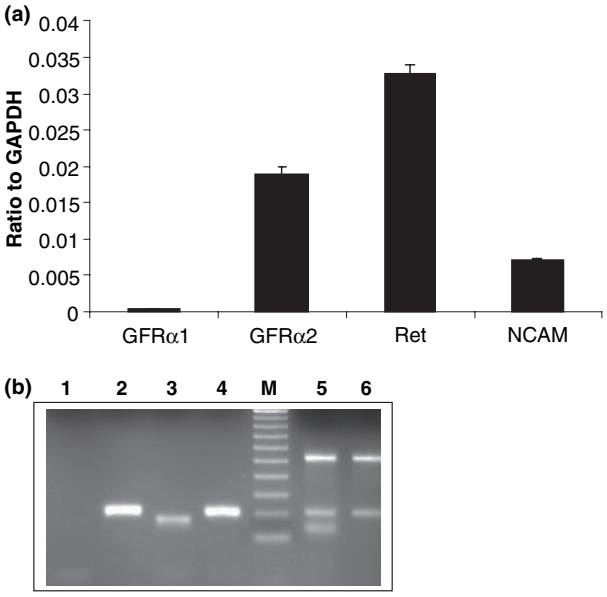

Fig. 1 Expression levels of GFRα, RET and NCAM transcripts in human neuroblastoma BE(2)-C cells by quantitative real-time PCR. (a) GFRα2, RET and NCAM were expressed at significant levels compared with GFRα1. The expression of GFRα1 was below the detection limit of the assay (< $1 : 10^6$, when expressed as the ratio of GFRα1 to GAPDH). The results are expressed as mean ± SEM of at least three independent experiments. (b) Amplification of GFRα1 (lane 1), GFRα2 (lane 2), RET (lane 3) and NCAM (lane 4) from BE(2)-C cells using primers as described in the Material and methods. Amplifications with primers designed to exons 1–4 of the GFRα2 gene using cDNA from human adult brain (lane 5) and BE(2)-C (lane 6) were carried out using primers (huGFRα2-1F: ATGATCTTGGCAAACGTCTT and huGFRα2-526R: TCTTGCAGTTGTCATTCAGGT). The amplified products and the 100 bp DNA marker (M) were resolved in a 2% agarose gel.

human brain, using GFRα2 primers designed to amplify exons 1–4 (Wong and Too 1998), showed multiple products. Three distinct products (about 500, 200 and 100 bp) were observed in human brain and only two of these (about 500 and 200 bp) were observed in BE(2)-C cells (Fig. 1b). These products were subsequently verified by sequencing to correspond to the exon organizations of previously known alternatively-spliced isoforms GFRα2a (526 bp), GFRα2b (211 bp) and GFRα2c (127 bp). The significant expressions of GFRα2, NCAM and RET in BE(2)-C cells thus provided a suitable model for further studies.

Regulation of MAPK (ERK1/2) phosphorylation by GDNF and NTN

Both GDNF and NTN activated MAPK (ERK1/2) rapidly in BE(2)-C cells (Fig. 2a). The responses to GDNF and NTN were similar in kinetics and sustainable over a period of 6 h (Fig. 2b). The MEK1/2 inhibitor, U0126, inhibited GDNF- and NTN-induced phosphorylation of MAPK (ERK1/2) in a dose-dependent manner (Fig. 2c). At the concentrations used, there was no evidence of cell death as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay (data not shown). This result suggests that GDNF and NTN activate MAPK signaling by phosphorylation on Thr202/204 of ERK1/2 through GFRα2.

Regulation of miRNA precursor expression by GDNF and NTN

In total, 23 pairs of pre-validated primers designed to anneal to the hairpin of miRNA precursors (Schmittgen *et al*. 2004) were used to quantify cDNA samples prepared from BE(2)-C cells. Initial attempts to co-reverse transcribe and accurately quantify both U6 and the miRNA precursors simultaneously were unsuccessful. The amplification of U6 from cDNA samples prepared from 1 μg RNA consistently showed Ct values of about 16 cycles (Fig. 3a). This is equivalent to the amplification of 10^7 copies of GFRα2, which has a similar amplicon size and PCR efficiency (3.5 cycles per log dilution). The failure to detect amplicon after 40 cycles (detection limit equivalent to one copy per reaction) defined the expression level of the miRNA precursors as undetectable. BE(2)-C cells were found to express eight of the 23 distinct miRNA precursors (miR-16, miR-18, miR-21, miR-24-2, miR-92-1, miR-93-1, miR-107 and miR-124a-2). All amplicons showed distinct melt curves (Fig. 3b) and the sizes were verified by gel electrophoresis (Fig. 3c). GDNF was found to transiently up-regulate the expressions of miR-21 and miR-24-2 precursors significantly (Fig. 4a). Interestingly, NTN was found only to down-regulate the expression of miR-92-1 precursor (Fig. 4b). No significant changes in the expression of the other miRNA precursors were observed.

To determine the contribution of the MAPK pathway in the regulation of the expression of miRNA precursors, U0126 was used to inhibit MEK1/2 activation (Fig. 5).

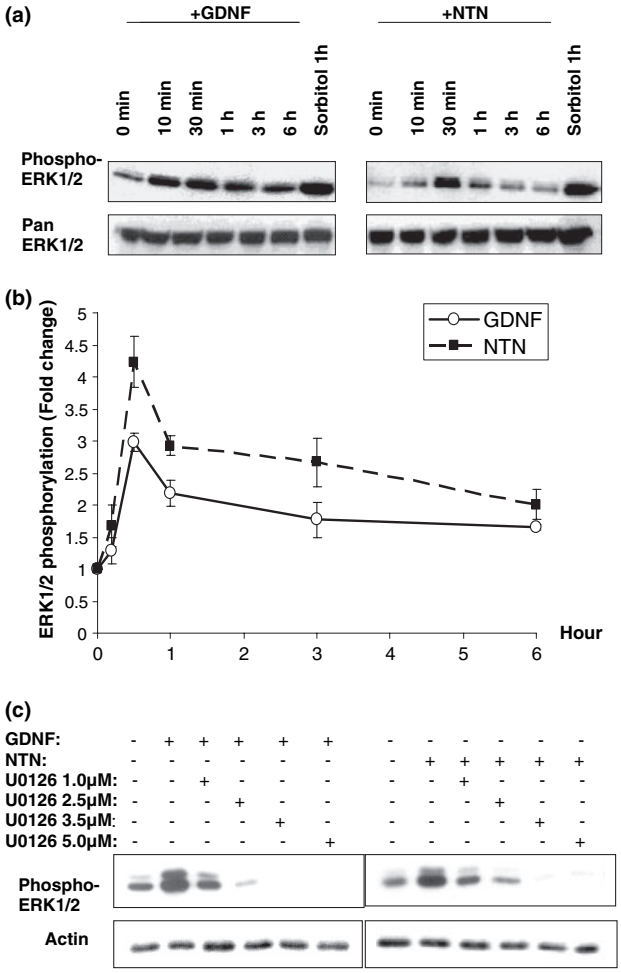

Fig. 2 GDNF and NTN induce MAPK (ERK1/2) phosphorylation in BE(2)-C cells. (a) Cells were stimulated with either GDNF or NTN and phosphorylated ERK1/2 was detected by western Blot. (b) Kinetic analyses of GDNF- and NTN-induced ERK1/2 phosphorylation. The blots were stripped and re-probed with anti-pan ERK1/2 antibody for the verification of protein loading (lower panels). (c) Concentration-dependent inhibition of MAPK activation by U0126 in GDNF- and NTN-stimulated BE(2)-C cells. The cells were pre-treated for 20 min with different concentrations of U0126 inhibitor before exposure to GDNF or NTN for a further 10 min. The results are expressed as standard deviations of triplicate measurements, and similar results were observed with three independent experiments.

At the submaximal dose (2.5 μM), U0126 inhibited the up-regulation of miR-21 (Fig. 5a) and miR-24-2 precursor expressions induced by GDNF (Fig. 5b), and the down-regulation of miR-92-1 precursor expression by NTN (Fig. 5c).

Differentiation of BE(2)-C cells with GDNF and NTN

Both miR-21 and miR-24-2 have previously been shown to be up-regulated in 12-O-tetradecanoylphorbol-13-acetate (TPA) differentiated HL-60 (Kasashima *et al*. 2004) and retinoic acid induced differentiation of embryonic stem cells

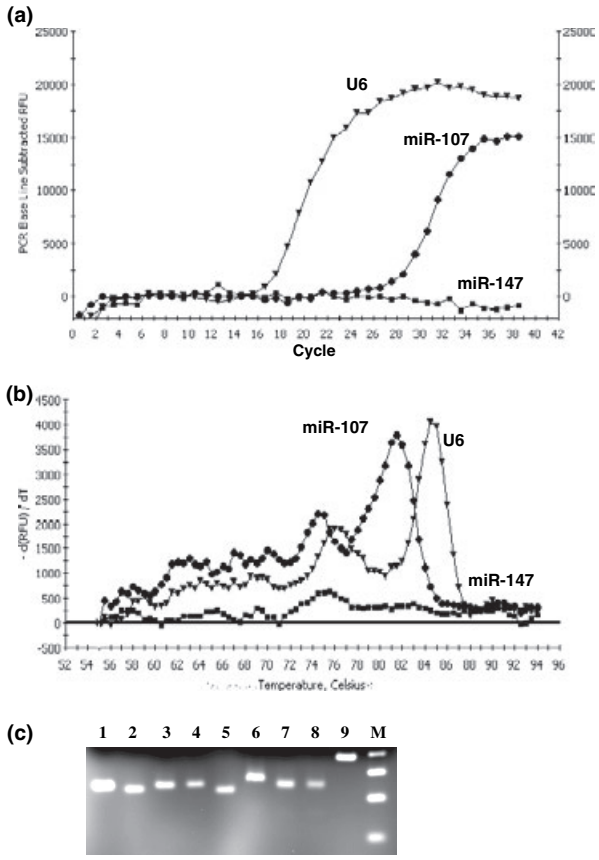

Fig. 3 Real-time PCR amplifications of miRNA precursors. Gene-specific primers designed to the hairpin of miR-107 and miR-147 precursors were used for amplification using cDNA samples prepared from BE(2)-C cells. (a) Real-time PCR quantification plot showing amplifications of U6 and miR-107. miR-147 amplicon was not detected, even after 40 cycles of amplification. No template controls showed background fluorescence, even after 40 cycles of amplification. (b) Melt-curve analysis after 40 cycles of amplification. Melt-curve analysis after amplifications showed distinct peaks of miR-107 and U6 products. (c) Gel electrophoresis of short hairpin products after amplification by real-time PCR. Amplifications were carried out using primers for the precursors of miR-107 (lane 1), miR-124a-2 (lane 2), miR-92-1 (lane 3), miR-93-1 (lane 4), miR-21 (lane 5), miR-24-2 (lane 6), miR-16 (lane 7), miR-18 (lane 8) and U6 (lane 9). The amplified products and the 25 bp DNA marker (M) were resolved in a 4% agarose gel. Similar results were obtained in at least three independent experiments.

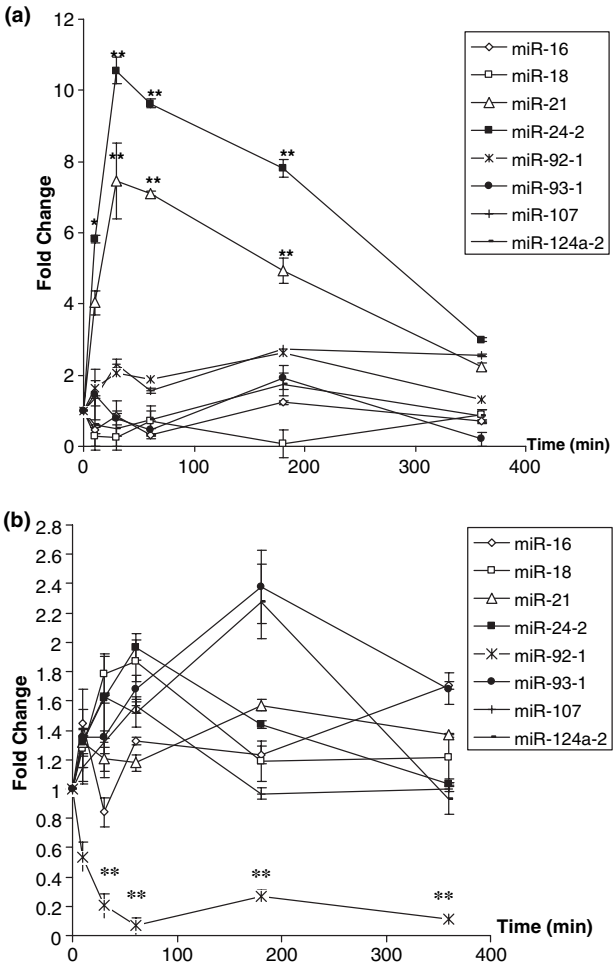

Fig. 4 Regulation of miRNA precursor expression by GDNF and NTN. miRNA precursor expression levels in BE(2)-C cells were expressed as fold change on stimulation with GDNF (a) and NTN (b) over a period of 6 h. Eight distinct miRNA precursors were detected in BE(2)-C cells, as shown in the inserts. Similar results were obtained from at least three independent experiments. Error bars indicate standard deviations of triplicate measurements. Significant differences in gene expression between ligand-stimulated and control cells were calculated using Student's paired *t*-test. A value of $p < 0.05$ was considered significant (**$p < 0.001$, *$p < 0.05$).

(Houbaviy *et al*. 2003). As these miRNA precursors were similarly up-regulated by GDNF in BE(2)-C cells (Fig. 4a), we examined the morphology of BE(2)-C cells when induced by GDNF and NTN over a period of 5 days. Differentiation of BE(2)-C cells was induced by retinoic acid but not GDNF or NTN (Fig. 6). The expression levels of miR-21 and miR-24-2 precursors in BE(2)-C cells were significantly increased by retinoic acid (Fig. 7). Interestingly, miR-92-1, which was down-regulated by NTN, was up-regulated by retinoic acid instead (Fig. 7).

Discussion

The results from this study reveal a novel function of GFRα2 in the regulation of miRNA precursors. This function is mediated by MAPK (ERK1/2), and the downstream regulation of distinct miRNA precursors is dependent on the ligand used.

GFRα2 and GFRα1 share about 48% amino acid identity and nearly complete conservation of cysteine residues, suggesting that these two receptors have similar three-dimensional structures and possibly share similar functions (Jing *et al*. 1997; Scott and Ibanez 2001). This is consistent

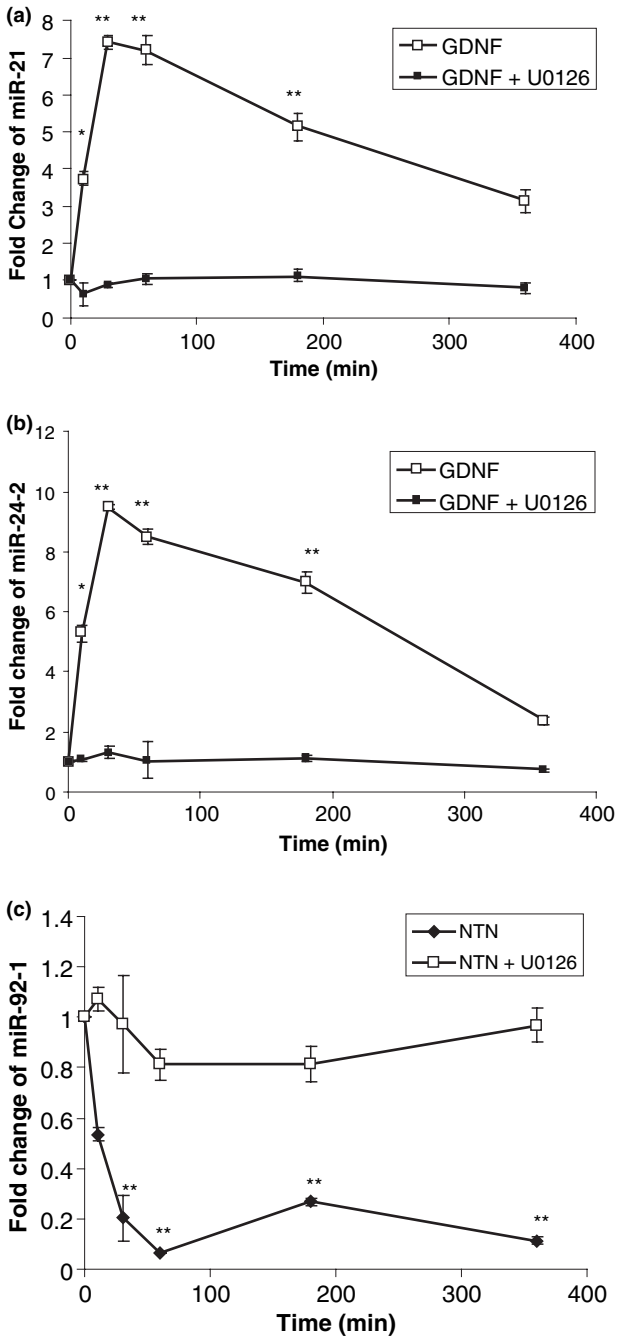

Fig. 5 Inhibition of miRNA precursor expression by U1026 in ligand-stimulated cells. Cells were pre-treated for 20 min with 2.5 μM U0126 before exposure to GDNF or NTN. The up-regulation of miR-21 (a) and miR-24-2 precursor (b) expression by GDNF was abolished in the presence of U0126. Similarly, the down-regulation of miR-92-1 by NTN (c) was abolished by U0126. The results were reproduced in at least three independent experiments. Error bars indicate standard deviations of triplicate measurements. Significant differences in the expression of the genes between ligand-stimulated and control samples were calculated using Student's paired *t*-test. A value of $p < 0.05$ was considered significant (**$p < 0.001$, *$p < 0.05$).

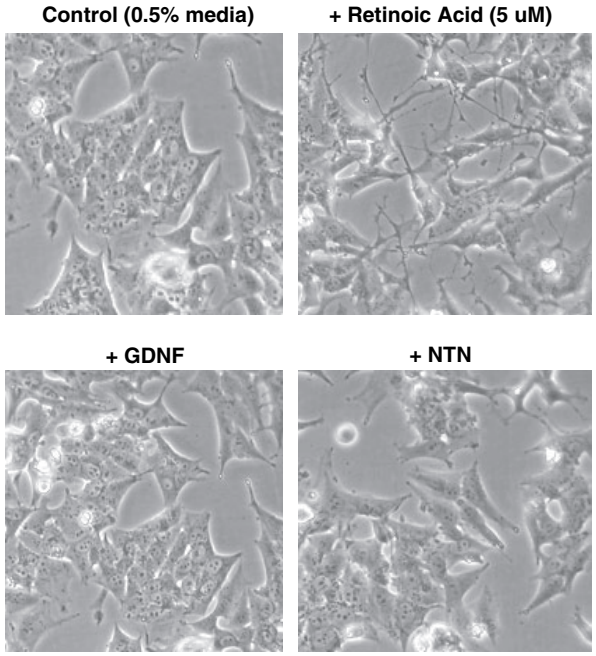

Fig. 6 Treatment of BE(2)-C cells with retinoic acid, GDNF and NTN. Cells (20 000) were seeded on 6-well plates overnight in DMEM supplemented with 10% FBS. Cells were then incubated in 0.5% FBS-supplemented medium, with or without all-*trans* retinoic acid (5 μM), GDNF (50 ng/mL) or NTN (50 ng/mL). The cells were then incubated for 3 days. Retinoic acid-treated cells, but not GDNF- or NTN-treated cells, showed neurite extension. The experiment was repeated at least three times with similar results.

with evidence from transfected cells and primary cultures, where GDNF and NTN have similar properties in activating the multi-component receptor complex (Baloh *et al*. 1997; Airaksinen *et al*. 1999; Wang *et al*. 2000; Scott and Ibanez 2001; Coulpier *et al*. 2002; Charlet-Berguerand *et al*. 2004). Furthermore, midbrain dopaminergic neurons, which only express GFRα1, appear to survive equally well with both GDNF and NTN *in vitro* and *in vivo* (Horger *et al*. 1998), and respond to neither ligand in the absence of GFRα1 (Cacalano *et al*. 1998). However, there are observations of distinct functional differences with the use of specific ligands. Although GDNF and NTN promote the survival of dopaminergic neurons through GFRα1 (Cacalano *et al*. 1998; Akerud *et al*. 1999), only GDNF possesses neuritogenic and hypertrophic effects (Akerud *et al*. 1999). In cultured sympathetic neurons, GDNF was able to promote the survival of cultured sympathetic neurons through GFRα2, but NTN could not promote survival through GFRα1 (Buj-Bello *et al*. 1997). Furthermore, GDNF, but not NTN, could promote the axonal growth of dorsal root ganglion (DRG) neurons through GFRα1 (Paveliev *et al*. 2004). Recently, GDNF and NTN have been shown to exhibit distinct and differential biochemical effects on cells

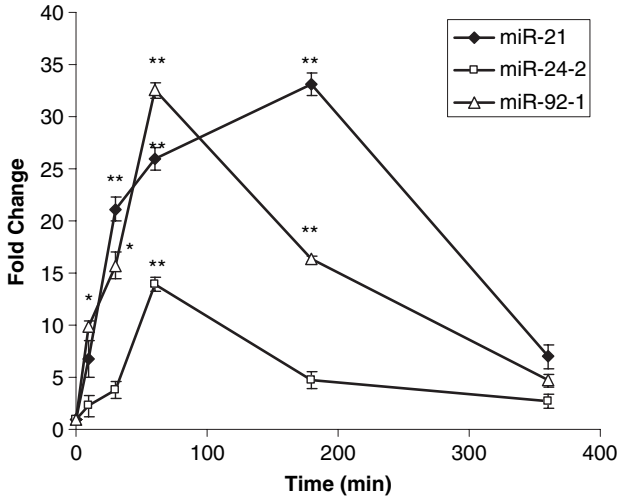

Fig. 7 Regulation of miRNA precursor expression in BE(2)-C by retinoic acid. The expression of miR-21, miR-24-2 and miR-92-1 precursors was up-regulated by retinoic acid over a period of 6 h. Similar results were obtained from at least three independent experiments. Error bars indicate standard deviations of triplicate measurements. Significant differences in expression of miRNA precursors between ligand-stimulated and control cells were calculated using Student's paired *t*-test. A value of $p < 0.05$ was considered significant ($^{**}p < 0.001$, $^{*}p < 0.05$).

expressing GFRα1 (Lee *et al*. 2006). The emerging view is that the crosstalk of exogenously applied GDNF and NTN with the non-preferred receptors may result in distinct functions.

In order to address the significance of GDNF and NTN crosstalk in regulating the expression of miRNA in a defined system, a cell that expresses the multi-component receptor complex with only one particular GFRα is required. The distinct advantage of using quantitative real-time PCR is that it allowed the reliable quantitative definitions of specificity, sensitivity and efficiency of an assay compared with the conventional end-point PCR-based assays (Wong and Medrano 2005). Using the highly specific, efficient and sensitive quantitative real-time PCR assays developed in this study, BE(2)-C cells were found to express NCAM, RET and GFRα2, but not GFRα1. These cells express GFRα2a and GFR2b, but not GFRα2c isoforms. The presence of GFRα2, but not GFRα1, in BE(2)-C cells agrees with the previous observation of Kobori *et al*. (2004) but not with a recent report using semi-quantitative PCR (Hansford and Marshall 2005). Consistent with the suggestion that both GDNF and NTN can activate the same multi-component receptor system, GDNF has been shown to induce the enhancement of phosphorylation and enzymatic activity of tyrosine hydroxylase through the activation of GFRα2 (Kobori *et al*. 2004).

GDNF and NTN are known to activate, in a similar manner, a number of signaling pathways, including ERK, phosphatidylinositol-3-kinase (PI3K)/AKT, p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) (Trupp *et al*. 1999; Takahashi 2001; Pezeshki *et al*. 2003; Ichihara *et al*. 2004), and regulate the expressions of various immediate early response genes (Fukuda *et al*. 2003; Pezeshki *et al*. 2003). Intriguingly, stimulation with GDNF and NTN resulted in similar kinetics of activation of ERK1/2 but regulated the expressions of distinct miRNAs. The rapid changes in gene expression of miR-21, miR-24-2 and miR-92-1 parallel the rapid induction of early response genes (Murphy *et al*. 2004; Sng *et al*. 2004). Interestingly, miR-92-1, which was rapidly down-regulated by NTN, was unaffected by GDNF stimulation but was found instead to be up-regulated by retinoic acid stimulation. Recently, neurotrophins have been shown to regulate the expression of a miRNA (miR-132) through a cAMP-dependent pathway, resulting in changes in neuronal morphology (Vo *et al*. 2005). The stimulation of GFRα2 by either GDNF or NTN did not result in neurite outgrowth in BE(2)-C cells. However, when stimulated by retinoic acid, these cells showed extensive neurite outgrowth and the up-regulation of miR-21, miR-24-2 and miR-92-1.

Analysis of the proximal sequences of miR-21, miR-24-2 and miR-92-1 by computational prediction of eukaryotic promoters (Scherf *et al*. 2000) revealed the existence of multiple regulatory elements, suggesting that the expression of these miRNAs may be regulated by multiple pathways, similar to other RNA polymerase II-mediated transcripts (Lee *et al*. 2004; Sng *et al*. 2004). The binding of NGF to TrkA receptor is known to activate two or more distinct signaling pathways, and the inhibition of a single pathway can inhibit the expression of the transcription of some genes (Marek *et al*. 2004). It is likely that the differential regulation of distinct miRNA expression by GDNF and NTN in BE(2)-C cells may similarly require the concerted signaling of multiple signaling pathways. The integration of signaling pathways regulating the expression of these miRNAs may provide a means for a more precise transcriptional control, depending on whether one or more pathways are activated. With the innumerable distinct signaling pathways induced by activated c-RET (Takahashi 2001; Ichihara *et al*. 2004), GDNF and NTN, acting through the same receptor complex, appear to activate two or more signaling mechanisms. The integration of these pathways is a subject for further investigation.

miRNAs are now thought to be involved in a number of physiological and developmental processes (Croce and Calin 2005; Harfe 2005; Miska 2005). Both miR-21 and miR-24-2 have been shown to be involved in cell proliferation and differentiation, and are overexpressed in various human cancers (Houbaviy *et al*. 2003; Kasashima *et al*. 2004; Chan *et al*. 2005; Iorio *et al*. 2005). These two miRNAs were up-regulated by GDNF and retinoic acid, but not NTN, in BE(2)-C cells. NTN, but not GDNF, rapidly down-regulate miR-92 expression in BE(2)-C cells. Interestingly, retinoic acid showed an opposite up-regulation of the same miRNA.

miR-92 has previously been shown to be amplified, overexpressed as a polycistronic miRNA cluster (Ota *et al*. 2004) and up-regulated in expression in some cancers (Calin *et al*. 2004). However, the function of miR-92 has yet to be determined.

The specific regulation of the expression of these miRNAs by GDNF and NTN suggests distinct functions associated with the activation of GFRα2. It is likely that many more miRNAs may be involved in various cellular processes, and that the expression of specific clusters of these miRNAs may be cell-type specific.

The search for miRNA targets by various algorithms (Lewis *et al*. 2003; John *et al*. 2004; Krek *et al*. 2005) has met with some success, and a recent study showed that some of the targets escape prediction (Nakamoto *et al*. 2005). To date, the specific targets of miR-21, miR-24-2 and miR-92-1 remain unknown, and predictions by various algorithms suggest more than a hundred putative targets for each of these microRNAs. In a continuing effort to further our understanding of the biochemical events underlying GLF and GFRα interactions, the quantitative changes in expression of other miRNA precursors (Jiang *et al*. 2005) and mature forms of miRNA (Chen *et al*. 2005; Raymond *et al*. 2005; Shi and Chiang 2005) are currently under investigation.

In summary, we have shown that GDNF and NTN specifically regulate the expression of distinct miRNAs through the same GFRα2 and multi-component receptor system. We have also provided the first evidence that the upstream regulation of the expression of some of these miRNAs by GDNF and NTN involves the same MAPK (ERK1/2) signaling pathway.

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Cellular/Molecular

Glial Cell Line-Derived Neurotrophic Factor and Neurturin Inhibit Neurite Outgrowth and Activate RhoA through GFRα2b, an Alternatively Spliced Isoform of GFRα2

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The glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) belong to a structurally related family of neurotrophic factors. NTN exerts its effect through a multicomponent receptor system consisting of the GDNF family receptor α2 (GFRα2), RET, and/or NCAM (neural cell adhesion molecule). GFRα2 is alternatively spliced into at least three isoforms (GFRα2a, GFRα2b, and GFRα2c). It is currently unknown whether these isoforms share similar functional and biochemical properties. Using highly specific and sensitive quantitative real-time PCR, these isoforms were found to be expressed at comparable levels in various regions of the human brain. When stimulated with GDNF and NTN, both GFRα2a and GFRα2c, but not GFRα2b, promoted neurite outgrowth in transfected Neuro2A cells. These isoforms showed ligand selectivity in MAPK (mitogen-activated protein kinase) [ERK1/2 (extracellular signal-regulated kinase 1/2)] and Akt signaling. In addition, the GFRα2 isoforms regulated different early-response genes when stimulated with GDNF or NTN. In coexpression studies, GFRα2b was found to inhibit ligand-induced neurite outgrowth by GFRα2a and GFRα2c. Stimulation of GFRα2b also inhibited the neurite outgrowth induced by GFRα1a, another member of the GFRα. Furthermore, activation of GFRα2b inhibited neurite outgrowth induced by retinoic acid and activated RhoA. Together, these data suggest a novel paradigm for the regulation of growth factor signaling and neurite outgrowth via an inhibitory splice variant of the receptor. Thus, depending on the expressions of specific GFRα2 receptor spliced isoforms, GDNF and NTN may promote or inhibit neurite outgrowth through the multicomponent receptor complex.

Key words: **GDNF; NTN; GFRα2; RhoA; inhibitory splice isoforms; neuroblastoma**

Introduction

Neurturin (NTN), glial cell line-derived neurotrophic factor (GDNF), Artemin, and Persephin are cysteine knot proteins and are members of the GDNF family ligands (GFLs) (Kotzbauer et al., 1996; Airaksinen and Saarma, 2002). These GFLs have been shown to support the growth, maintenance, and differentiation of a wide variety of neuronal and extraneuronal systems (Saarma and Sariola, 1999). Each GFL is known to bind preferentially to one GDNF family receptor α (GFRα) *in vitro*, and the activation of the multicomponent receptor system shows some degree of promiscuity in their ligand specificities (Horger et al., 1998; Airaksinen et al., 1999; Cik et al., 2000; Wang et al., 2000; Scott and Ibanez, 2001). NTN is thought to signal through its preferred receptor complex consisting of GFRα2, RET, and/or neural cell adhesion molecule (NCAM) (Baloh et al., 1997; Buj-Bello et al., 1997; Widenfalk et al., 1997; Paratcha et al., 2003).

Alternative splicing is prevalent in many mammalian genomes, as a means of producing functionally diverse polypeptides from a single gene (Blencowe, 2006). It has been estimated that >50% of human multi-exon genes are alternatively spliced (Modrek and Lee, 2002). Multiple alternatively spliced variants of GFRα1 (Sanicola et al., 1997; Dey et al., 1998; Shefelbine et al., 1998), GFRα2 (Wong and Too, 1998; Dolatshad et al., 2002), and GFRα4 (Lindahl et al., 2000, 2001; Masure et al., 2000) have been reported. Similarly, alternative spliced isoforms of the coreceptors RET (Lorenzo et al., 1997; de Graaff et al., 2001; Lee et al., 2002) and NCAM (Povlsen et al., 2003; Buttner et al., 2004) have been reported. The alternatively spliced isoforms of GFRα1 have recently been shown to exhibit distinct biochemical functions (Charlet-Berguerand et al., 2004; Yoong et al., 2005). These observations are consistent with the emerging view that the combinatorial interactions of the spliced isoforms of GFRα, RET, and NCAM may contribute to the multicomponent signaling system to produce the myriad of observed biological responses.

We have previously shown that all three isoforms of GFRα2 are expressed at significant levels in the murine whole brain and embryo (Too, 2003). It is, however, unknown whether these isoforms serve distinct or redundant functions. To gain a better insight into their biological relevance in the CNS, the expression levels of the isoforms in different regions of the human brain were quantified by highly specific real-time PCR assays. The biological

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functions of the isoforms were then examined in a neuronal differentiation model using Neuro2A cells and in BE(2)-C cells, which express the spliced isoforms endogenously.

Here, we showed that ligand activation of the isoforms differentially activated mitogen-activated protein kinase (MAPK) [extracellular signal-regulated kinase 1/2 (ERK1/2)] and AKT signaling and regulated distinct early-response genes. Furthermore, both GDNF and NTN induced neurite outgrowth through GFRα2a and GFRα2c, but not GFRα2b. Activation of GFRα2b inhibited neurite outgrowth induced by the other two GFRα2 isoforms as well as GFRα1a and retinoic acid. RhoA was also found to be activated by GDNF and NTN through GFRα2b. This study thus provides the first piece of evidence of a dominant inhibitory activity of GFRα2b on neurite outgrowth and distinct signaling mechanisms underlying the activation of spliced isoforms.

Materials and Methods

Cell culture. Neuro2A (catalog #CCL-131; American Type Culture Collection, Manassas, VA) and BE(2)-C (catalog #CRL-2268; American Type Culture Collection) cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT). All cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C.

Reverse transcription reaction. Total RNA for different human brain regions was purchased from Clontech (Palo Alto, CA). Total RNA from Neuro2A cells was prepared as described previously (Too and Maggio, 1995). The integrity of isolated total RNA was validated by denaturing agarose gel electrophoresis. Five micrograms of total RNA were reverse transcribed using 400 U of ImpromII and 0.5 μg of random hexamer (Promega, Madison, WI) for 60 min at 42°C according to the manufacturer's instructions. The reaction was terminated by heating at 70°C for 5 min, and the cDNA was used directly for quantitative real-time PCR. Three independent preparations of cDNA were used for the study. All measurements were performed in triplicate.

Plasmids constructions. To prepare plasmid standards for quantitative real-time PCR, open reading frames of human GFRα2 isoforms and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were subcloned into p-GEMT (Promega). For early-response genes and transcriptional factors, partial sequences were subcloned using the same primers used for real-time PCR quantification. *Xba*I or *Xmn*I (Promega) was used to linearize plasmids to be used as templates for real-time PCR amplifications.

Sequence-independent real-time PCR. Real-time PCR was performed on the iCycler iQ (Bio-Rad, Hercules, CA) using SYBR Green I. The threshold cycles (Ct) were calculated using the Optical interface version 3.0B. Real-time PCR was performed after an initial denaturation for 3 min at 95°C, followed by 40–60 cycles of 60 s denaturation at 95°C, 30 s annealing at 60°C, and 60 s extension at 72°C. Fluorescent detection was performed at the annealing phase. The reaction was performed in a total volume of 50 μl in 1× XtensaMix-SG (BioWORKS, Singapore), containing 2.5 mM $MgCl_2$, 10 pmol of primer, and 1.25 U of Platinum DNA polymerase (Invitrogen, Carlsbad, CA). Melt-curve analyses were performed at the end of PCR to verify the identity of the products. A specific exon-overlapping forward primer used for amplification of human GFRα2a was designated as "2a 15 + 9F" (5′-TCTTCTTCTTTCTAGACGAGACCC-3′), for human GFRα2b as "2b 17 + 7F" (5′-CCTCTTCTTCTTTCTAGGTGAGGA-3′), and for human GFRα2c as "2c 18 + 5F" (5′-GCCTCTTCTTCTTTCTAGGGACA-3′). A common reverse primer, designated as "553R" (5′-GCAGATGGAGATGTAGGAGGAG-3′), was used for all three isoforms. The primer pair 5′-GATCATCAGCAATGCCTCCT-3′ and 5′-GCCATCACGCCACAGTTT-3′ was used to amplify human GAPDH. All real-time PCR quantification was performed simultaneously with linearized plasmid standards and a nontemplate control. The gene expression levels were interpolated from standard curves and normalized to the expressions of GAPDH in the same samples. Differences in the expression levels of GFRα2 isoforms were analyzed using the paired Student's *t* test with a level of significance of $p < 0.05$.

Generation of Neuro2A cells expressing GFRα2 isoforms. The murine neuroblastoma cell line Neuro2A, which express endogenous RET and NCAM, was stably transfected with murine GFRα2a, GFRα2b, GFRα2c, or vector control pIRESneo (Clontech) using Fugene-6 (Roche, Mannheim, Germany) and selected with 0.8 mg/ml G418 (Promega), over a period of 2 months. Primers used for measuring GFRα2 isoforms, RET, and NCAM expression were as described previously (Too, 2003; Yoong et al., 2005). For coexpression studies, GFRα2a, GFRα2c, or GFRα1a was cloned into the proximal 5′ multiple cloning site, whereas GFRα2b was cloned into a distal 3′ multiple cloning site of the bicistronic pIRES vector (Clontech). All studies were performed with three independent clones.

Assessment of neurite outgrowth in GFRα2-transfected Neuro2A cells. Twenty thousand to 50,000 cells per well were seeded on six-well plates overnight, in DMEM supplemented with 10% FBS. Cells were then incubated with medium containing 0.5% FBS, with or without 50 ng/ml recombinant human GDNF (Biosource, Camarillo, CA) or NTN (ProSpec-Tany TechnoGene, Rehovot, Israel). Cells were incubated for 3 more days. All-*trans* retinoic acid (5 μM; Sigma, St. Louis, MO) was used as a positive control for inducing neurite outgrowth. Cells bearing at least one neurite twice the length of the cell bodies were scored. More than 600 cells from three different fields were counted per well. Statistical significance between ligand-stimulated and control samples was calculated using the paired Student's *t* test. A value of $p < 0.05$ was considered significant.

Immunocytochemistry and confocal microscopy. Cells were seeded on chamber slides, fixed with 4% paraformaldehyde in 1× PBS for 15 min at 37°C, and subsequently fixed in methanol at −20°C for an additional 15 min. After three washes with 1× PBS, cells were permeabilized and blocked with serum (1:10; Dako, Glostrup, Denmark) and 0.5% Triton X-100 in 1× PBS for 30 min at room temperature. The cells were then incubated with F-actin (phalloidin-conjugated tetramethylrhodamine isothiocyanate) and high-molecular-weight neurofilament protein (NF-200) antibody (Sigma) in 0.1% Triton X-100, 0.1% BSA, and 1× PBS for 1 h at 37°C and washed three times in 1× PBS. A secondary antibody (Alexa Fluor 488; Invitrogen, Eugene, OR) was then added at a dilution of 1:200 and incubated for 1 h. The cells were then washed in 1× PBS and mounted for visualization. Image acquisition was obtained using a Zeiss (Oberkochen, Germany) 510 meta confocal microscope equipped with fluorescence detection.

Immunoblotting. Phosphorylation of MAPK (ERK1/2) or Akt was analyzed as follows. Cells were initially seeded in DMEM with 10% FBS for 24 h, and serum was depleted (0.5% FBS) for 16 h. The cells were then treated with 50 ng/ml GDNF, NTN, Artemin, or Persephin (PreproTech, London, UK) in serum-free medium for different periods of time at 37°C. For dose–response studies, cells were stimulated with different concentrations of ligands for 10 min at 37°C. Control treatment with 1 M Sorbitol (Sigma) was performed simultaneously. The supernatants were then removed, and cells were washed once with 1× PBS and subsequently lysed in 2% SDS. Protein concentrations were estimated using the BCA assay (Pierce, Rockford, IL). ERK1/2 or Akt phosphorylation was analyzed by Western blot using phospho-specific antibodies according to the manufacturer's instructions (Cell Signaling Technology, Danvers, MA). Blots were stripped with Restore Western Stripping Buffer (Pierce) and reprobed with pan antibodies to verify equal loading of protein.

For studying kinetics and dose–response of ligand-induced ERK1/2 activation, dot blot analysis was performed using the BIO Dot Apparatus (Bio-Rad). Five micrograms of protein were loaded per well in triplicates. Blots were then detected by anti-phospho ERK1/2 antibodies (Cell Signaling Technology) according to the manufacturer's instructions. Densities of blots were imaged and measured by Quantity One 4.0 (Bio-Rad).

Binding of [^{125}I]GDNF to GFRα2 isoforms transfected Neuro2A cells. [^{125}I] GDNF (~1000 mCi/mmol) was prepared using Bolton and Hunter reagent (Amersham Biosciences, Piscataway, NJ). Briefly, 10 μg of recombinant human GDNF (Biosource) was labeled with 1 mCi of Bolton and Hunter reagent for 1 h at room temperature according to the

manufacturer's instructions. The reaction was then terminated by adding 10 μl of 0.1% tyrosine. Radiolabeled GDNF was then purified on a Sephadex G-10 column.

Binding studies were performed as described previously (Jing et al., 1997). Briefly, 0.1 million cells were seeded per well on 24-well Costar (Cambridge, MA) tissue culture plates for 2 d before the assay. Before the experiment, cells were placed on ice for 15–20 min and washed once with ice-cold DMEM buffer and 25 mM HEPES, pH 7.0. Cells were then incubated at 4°C for 3 h with 0.2 ml of binding buffer [DMEM, 25 mM HEPES, 2 mg/ml bovine albumin serum, and Complete Inhibitor Cocktail (Roche), pH 7.0] containing 50 pM [^{125}I]GDNF and various concentrations of unlabeled GDNF. At the end of incubation, cells were washed three times with 0.3 ml of ice-cold washing buffer and lysed in 0.1% SDS containing 1 M NaOH. The radioactivity in lysates was measured using the auto gamma counter (PerkinElmer Packard, Wellesley, MA).

Measurements of early-response genes regulated by GDNF and NTN. Cells were seeded in DMEM with 10% FBS for 24 h, followed by serum depletion (0.5% FBS) for 18–24 h. The cells were then treated with GDNF (50 ng/ml) or NTN (50 ng/ml) in serum-free medium for varying periods of time at 37°C. Total RNA was then isolated and reverse transcribed as described above. The gene expression levels were then quantified by real-time PCR using gene-specific primers. Primers used for amplification of early response genes were as follows: EGR-1-328F/EGR-1-459R (5′-GAGAAGGCGATGGTGGAGACGA-3′/5′-GCTGAAAAGGGGTTCAGGCCA-3′) for *egr-1*; EGR-2-1F/EGR-2-179R (5′-ATGAACGGAGTGGCGGGAGAT-3′/5′-TCTGGATAGCAGCTGGCACCAG-3′) for *egr-2*; mcfos(B)651F/mcfos(B)901R (5′-TGTGGCCTCCCTGGATTT-3′/5′-CTGCATAGAAGGAACCGGAC-3′) for *c-fos*; and mFosB(A)1926F/mFosB(A)2107R (5′-CAGGGTCAACATCCGCTAA-3′/5′-GGAAGTGTACGAAGGGCTAACA-3′) for *fosB*. Expression of target genes and GAPDH was interpolated from standard curves. The fold change of each target gene was calculated as a change in gene expression of the stimulated sample normalized to GAPDH compared with gene expression of the control sample normalized to GAPDH.

Silencing of GFRα2b in BE(2)-C. Small interfering RNA (siRNA) duplexes (Invitrogen) were designed across specific exon (exons 1 and 3) boundaries of GFRα2b (siGFRα2b-15+5: TCTTCTTCTTTCTAGGTGAG; siGFRα2b-13+7: TCTTCTTCTTTCTAGGTGAGGA; siGFRα2b-10+10: TTCTTTCTAGGTGAGGAGTT; siGFRα2b-7+13: TTTCTAGGTGAGGAGTTCTA; siGFRα2b-5+15: TCTAGGTGAGGAGTTCTACG). Subconfluent cells (50–80%) were seeded on six-well plates, in 10% FBS DMEM. Cells were transfected with siRNA duplexes (20 pmol) using Transfectin (Bio-Rad) in 400 μl of 0.5% FBS DMEM per well. Total RNA was isolated 6 h after transfection, and gene expression was measured by real-time PCR. For differentiation studies using BE(2)-C, 6 h after silencing of GFRα2b, 2 ml of differentiation medium containing retinoic acid (5 μM), GDNF (50 ng/ml), or NTN (50 ng/ml) in 0.5% FBS DMEM was added to the medium. Analyses of morphological differences were performed after 3 d.

RhoA assay. Neuro2A cells were seeded in 10% FBS DMEM and incubated for 18–24 h. Subsequently, the serum was reduced to 0.5% in DMEM, and the cells were incubated for an additional 18–24 h. Cells were then treated with 10 μM lysophosphatidic acid (LPA; Sigma), GDNF (50 ng/ml), or NTN (50 ng/ml) in serum-free DMEM for 10 min. Cells were lysed and used directly for the GTP-RhoA pull-down assay according to the manufacturer's instructions (Pierce). RhoA inhibitor exoenzyme C3 transferase and Rho kinase (ROCK) inhibitor Y27632 were purchased from Calbiochem (La Jolla, CA). Exoenzyme C3 transferase was transfected into cells using the lipotransfecting agent Transfectin (Bio-Rad), at 1 μl of Transfectin/1 μg of C3 transferase per well of a six-well plate, 4 h before start of the experiment. Cells were then treated with RhoA inhibitor exoenzyme C3 transferase (1 μg/ml) or ROCK inhibitor Y27632 (10 μM), in the presence or absence of differentiating stimuli. LPA (10 μM; Sigma) was used as a positive control for activities of Rho and ROCK inhibitor.

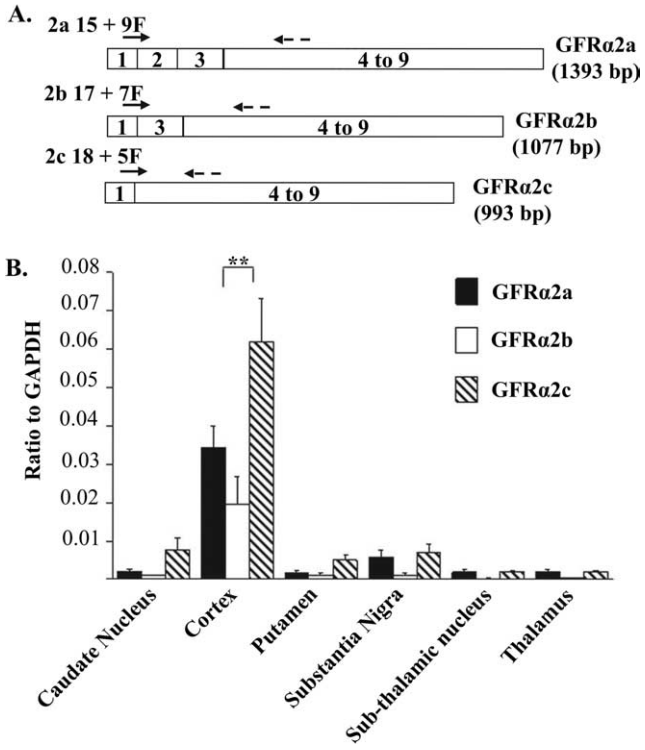

Figure 1. Real-time PCR quantification of the expressions of GFRα2 isoforms in the human brain. ***A***, A schematic diagram showing the protein coding exons and the positions of the primers used for quantitative real-time PCR. Exons 1–9 encode the full-length protein sequence of GFRα2a. Specific forward primers (2a 15 + 9F for GFRα2a, 2b 17 + 7F for GFRα2b, and 2c 18 + 5F for GFRα2c) were designed across exon junctions, whereas a common reverse primer (553R) was used for the amplification of all the three isoforms. ***B***, The expression levels of GFRα2 isoforms in a different human brain region normalized to the levels of GAPDH in the same tissue. The results were expressed as mean ± SEM ($n = 3$). Significant differences between the expressions of the isoforms were calculated using paired Student's *t* test. A value of $p < 0.05$ was considered significant (**$p = 0.001$).

Results

Differential expression profiles of GFRα2 spliced variants

Currently, the expression levels of GFRα2 spliced variants in specific regions of the brain are unknown. To address this issue, we have developed sequence-independent real-time PCR assays to quantify each of the spliced variants with high specificity and sensitivity.

To discriminate between the three spliced variants of human GFRα2, overlapping exon primers were designed across exons 1 and 2, 1 and 3, or 1 and 4 to enable the specific detection and quantification of GFRα2a, GFRα2b, and GFRα2c, respectively (Fig. 1*A*). Because the amplification products of GFRα2a (545 bp), GFRα2b (233 bp), and GFRα2c (172 bp) were different in sizes, it was critical to determine the optimal cycling parameters for the selective amplification of each of the transcripts. A dwell time of 30 s for annealing, 60 s for denaturation at 95°C, and 60 s for extension at 72°C were found to be optimal for the amplifications of all three isoforms. The slopes of the plots of Ct versus $\log_{10}$ mole of the human GFRα2a, GFRα2b, and GFRα2c standards were 3.37 ± 0.30 ($r^2 = 0.98$), 4.12 ± 0.41 ($r^2 = 0.99$), and 3.82 ± 0.54 ($r^2 = 0.99$), respectively. The samples diluted in parallel with the standards (data not shown). The specificity of amplifying a particular isoform compared with the other variants was $>10^6$-fold (data not shown). Hence, the amplifications of GFRα2b and GFRα2c were at least 10^6-fold less efficient than amplifying GFRα2a, when using GFRα2a exon-overlapping

primers. The detection limits of the assays were estimated to be <100 copies of transcripts per reaction.

Using these highly sensitive and specific assays, the expression levels of the GFRα2 alternatively spliced isoforms were quantified in caudate nucleus, cortex, putamen, substantia nigra, subthalamic nucleus, and thalamus of the human brain (Fig. 1*B*). The three GFRα2 isoforms were detected at significant levels ($>10^4$ copies per reaction) in all areas of the brain, with expression levels highest in the cortex. In cortex, all three isoforms were expressed at comparable levels, with GFRα2b expression significantly lower than GFRα2c ($p < 0.01$).

GFRα2 isoforms differentially activated ERK1/2 and Akt

To investigate the biological significance of alternatively spliced GFRα2 isoforms, stable transfectants were generated in Neuro2a cells. We have shown previously that Neuro2a cells express RET and NCAM, but not GFRα2 receptors, endogenously (Yoong et al., 2005). The expression levels of GFRα2 isoforms in stably transfected Neuro2a cells (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) were comparable to that expressed in the human cortex (Fig. 1*B*).

When stimulated with NTN, all the three isoforms induced the rapid phosphorylation of ERK1/2 (Fig. 2*A*). However, when stimulated with GDNF, GFRα2a (Fig. 2*A*,*C*) and GFRα2c (Fig. 2*A*,*E*), but not GFRα2b (Fig. 2*A*,*D*), induced significant ERK1/2 phosphorylation (more than twofold). The extent of ERK1/2 phosphorylation was similar when GFRα2a (Fig. 2*C*) and GFRα2c (Fig. 2*E*) activated with either GDNF or NTN. However, GFRα2b showed rapid and significant phosphorylation of ERK1/2 only with NTN stimulation but not by GDNF (Fig. 2*A*,*D*). Both GDNF and NTN induced ERK1/2 phosphorylation in a dose–response manner in GFRα2a (Fig. 2*F*) and GFRα2c (Fig. 2*H*) transfectants. GDNF appeared to be slightly more potent than NTN in inducing ERK1/2 phosphorylation in both transfectants (Fig. 2*F*,*H*). Compared with the stimulation with NTN, GFRα2b when stimulated with GDNF showed no significant increase in ERK1/2 phosphorylation even at the highest dose (Fig. 2*G*). No significant increase in the phosphorylation of ERK1/2 was observed in vector (pIRESneo) control transfected Neuro2A cells when stimulated with either GDNF or NTN (data not shown).

We next investigated the ligand-regulated phosphorylation of Akt using GDNF or NTN (Fig. 2*B*). NTN induced rapid and significant phosphorylations of Akt in all three isoform transfectants. However, GDNF induced the rapid and significant phosphorylations of Akt in cells expressing GFRα2b and GFRα2c.

The other GFLs, Artemin and Persephin, did not induce significant phosphorylation of ERK1/2 or Akt in any of the GFRα2

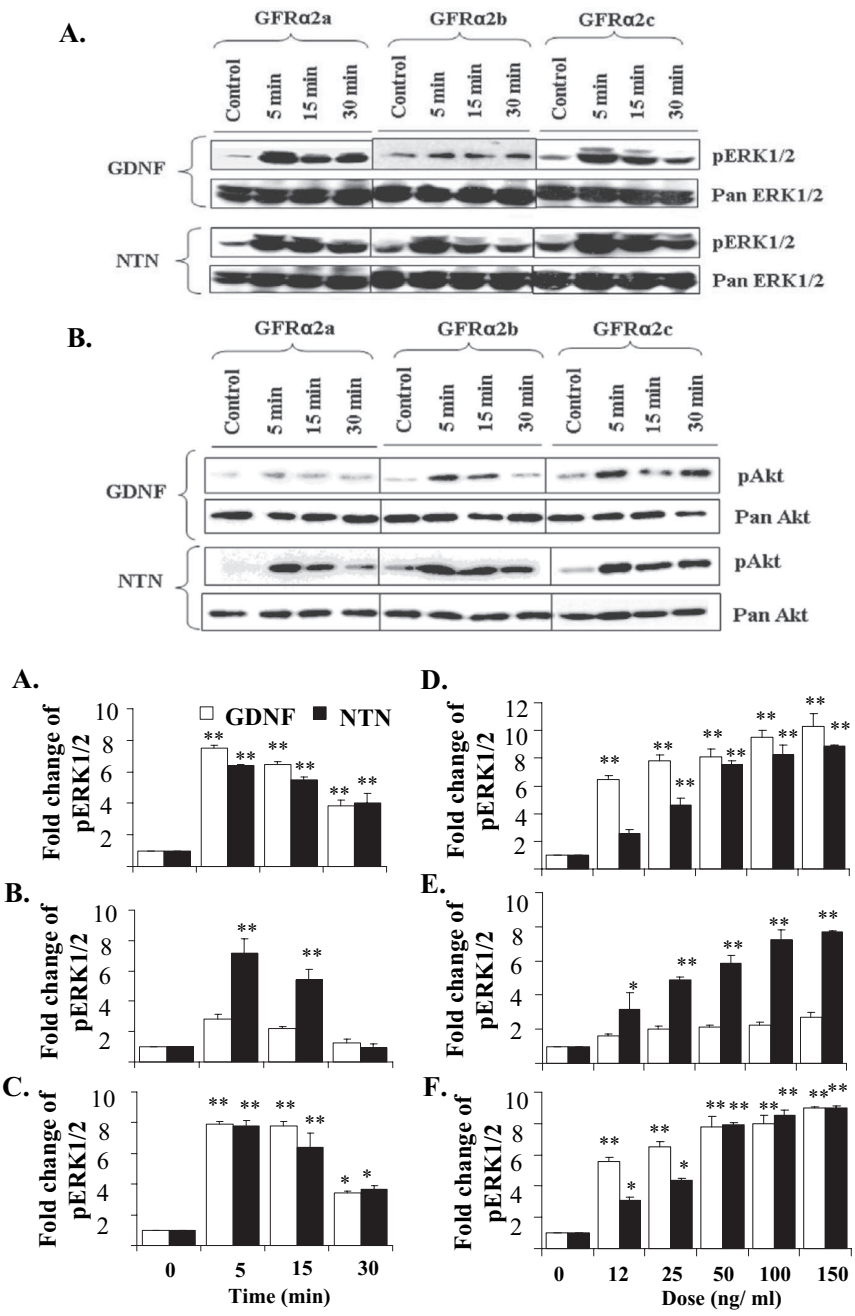

Figure 2. Activations of ERK1/2 and Akt in GFRα2 isoforms transfected Neuro2A cells when stimulated by either GDNF or NTN. Cells were stimulated in serum-free medium, with or without GDNF or NTN (50 ng/ml), for the period of time indicated. ***A***, ***B***, Five micrograms of protein were loaded and separated by SDS electrophoresis, and phosphorylated ERK1/2 (pERK1/2; ***A***) or Akt (pAkt; ***B***) was then detected by Western blot. Blots were stripped and reprobed with pan antibody as loading controls. ***C–E***, Kinetics of GDNF and NTN induced ERK1/2 activations in GFRα2a (***C***), GFRα2b (***D***), and GFRα2c (***E***). Cells were treated with 50 ng/ml GDNF or NTN for 5, 15, and 30 min. ***F–H***, Dose responses of the activation of ERK1/2 when stimulated with GDNF or NTN in GFRα2a (***F***), GFRα2b (***G***), and GFRα2c (***H***) isoforms. Cells were stimulated for 10 min with ligand at various doses. For kinetic and dose–response studies, 5 μg of protein was loaded per well for dot blot quantification of phospho-ERK1/2 (pERK1/2). The means ± SD were calculated from results obtained in triplicates. Significant differences in fold change of pERK1/2 between ligand stimulated and control were calculated using the paired Student's *t* test. A value of $p < 0.05$ was considered significant (**$p < 0.001$; *$p < 0.05$). Experiments were repeated three times with two independent clones with similar results.

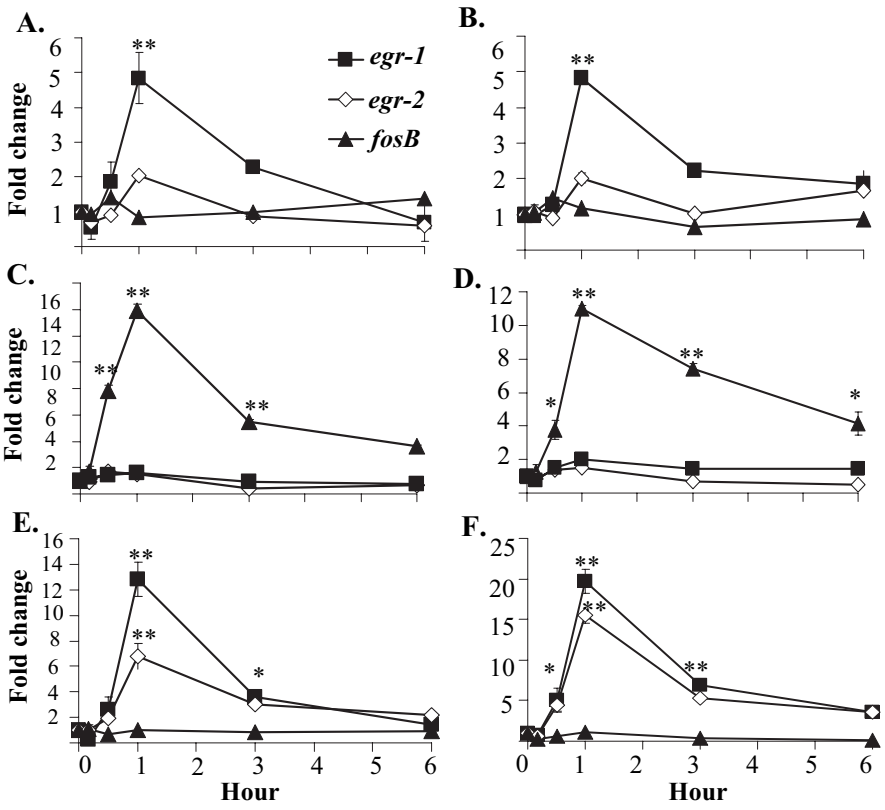

Figure 3. Kinetic analyses of the regulations of early-response genes by GDNF and NTN in GFRα2 isoform transfectants. The fold change of mRNA expressions of early-response genes in cells expressing GFRα2a (***A***), GFRα2b (***C***), and GFRα2c (***E***) when stimulated with GDNF and GFRα2a (***B***), GFRα2b (***D***), and GFRα2c (***F***) when stimulated with NTN at the designated period of time is shown. The expression levels were measured by quantitative real-time PCR. Similar results were obtained from more than three separate experiments. Error bars indicate SDs of triplicate measurements from one study. Significant differences in expression of genes between ligand stimulated and control were calculated using the paired Student's *t* test. A value of $p < 0.05$ was considered significant (**$p < 0.001$; *$p < 0.05$).

isoform transfectants (data not shown). In addition, neither GDNF nor NTN was found to activate p38 and c-Jun N-terminal kinase (JNK) in any of the GFRα2 isoform transfectants, even at concentrations as high as 100 ng/ml and over a period of 1 h of ligand stimulation (data not shown).

[^{125}I]GDNF bound equally well to all three GFRα2 isoforms

NTN has been shown to bind with similar affinities to the GFRα2 isoforms (Scott and Ibanez, 2001). Because GDNF failed to induce a significant increase in the phosphorylation of ERK1/2 in GFRα2b transfectants (Fig. 2*A*,*D*,*G*), it is possible that GDNF may not bind to this isoform. To address this possibility, we next performed a ligand displacement study using [^{125}I]GDNF (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). GDNF displaced the binding of [^{125}I]GDNF to the three GFRα2 isoforms with similar potencies. The IC_{50} for the displacements of cells transfected with GFRα2a, GFRα2b, and GFRα2c were 3.27 ± 0.02, 2.79 ± 0.16, and 2.31 ± 0.09 nM (mean ± SD), respectively. Parental Neuro2A or cells transfected with pIRESneo showed no significant binding to [^{125}I]GDNF. This result indicates that GDNF binds to all three isoforms with similar affinities.

GFRα2 isoforms activated different transcriptional genes

The differential activation of ERK1/2 and Akt (Fig. 2) suggests the possibility that downstream biochemical mechanisms may differ. To explore this issue, we measured the changes in gene expression of the *fos* family (c-*fos*, *fosB*), *jun* family (c-*jun, jun-b*), *egr* family (*egr1–4*), and GDNF-inducible transcription factors *mGIF* and *mGZF1* in response to GDNF and NTN (supplemental Table 1, available at www.jneurosci.org as supplemental material). These factors have previously been shown to be activated with GDNF or NTN (Yajima et al., 1997; Trupp et al., 1999; Kozlowski et al., 2000; Fukuda et al., 2003; Pezeshki et al., 2003). The kinetics of gene activations over a period of 6 h was quantified by real-time PCR (Fig. 3). Distinct ligand-induced early-response gene expressions were observed with the activation of the different GFRα2 isoforms. GFRα2a, when stimulated by GDNF (Fig. 3*A*) or NTN (Fig. 3*B*), upregulated *egr-1* by as much as fourfold to fivefold. GFRα2b, when stimulated by GDNF (Fig. 3*C*) or NTN (Fig. 3*D*), upregulated *fosB* by >10-fold compared with control. When stimulated with GDNF (Fig. 3*E*) or NTN (Fig. 3*F*), GFRα2c upregulated the expressions of *egr-1* and *egr-2*. With the other genes, no significant changes were observed with GDNF or NTN stimulations. These results showed that the activation of GFRα2b isoform regulates the transcription of specific sets of early-response genes.

Neurite outgrowths were induced by GFRα2a and GFRα2c, but not GFRα2b

Neuro2a cells serve as an excellent *in vitro* model system for studying signaling pathways mediating neurite outgrowth. Under normal growth conditions, most Neuro2a cells spontaneously sprout a basal level of neurites. However, treatment with a variety of stimuli cause these cells to develop extensive neurites similar to changes observed in hippocampal and cortical cultures (Ahmari et al., 2000; Washbourne et al., 2002).

To investigate possible morphological changes induced by the activation of the GFRα2 isoforms, the transfectants were stimulated with either GDNF or NTN. Both GFRα2a and GFRα2c transfectants showed extensive neurite outgrowths when stimulated with either ligand, comparable to the effects of retinoic acid (Fig. 4). Unexpectedly, neither NTN nor GDNF induced neurite outgrowth in cells expressing GFRα2b (Fig. 4). Immunocytochemical staining for β-III tubulin confirmed these observations. (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Cells expressing GFRα2b extended neurite-like structures when treated with retinoic acid, indicative of the potential for neurite outgrowth (Fig. 4*A*). GDNF and NTN have no neuritogenic effect on control vector-transfected Neuro2A cells (Fig. 4).

To further examine the morphological changes in these cells, two major cytoskeletal components, F-actin and high-molecular-weight neurofilament protein (NF-H), which are involved in neurite outgrowth dynamics, were visualized by fluorescent staining (Myers et al., 2006). With ligand (GDNF or NTN)-stimulated GFRα2a and GFRα2c transfectants, NF-H-positive

filopodia (axon-like processes) were relatively long and formed thick threads. Protrusions with F-actin staining were observed at the edges of the thick NF-H-positive axon-like elements and cell bodies (supplemental Fig. 4*C*, arrowheads, available at www.jneurosci.org as supplemental material). Engorgements were seen at some terminal structures that were both NF-H and F-actin positive (supplemental Fig. 4*C*, arrow, available at www.jneurosci.org as supplemental material). Long extensions were not obvious with cells expressing GFRα2b when stimulated with either ligand. Instead, F-actin-positive staining was found at the periphery of these cells where NF-H was not found to colocalize extensively (supplemental Fig. 4*F*, available at www.jneurosci.org as supplemental material). These observations provide additional evidence of the lack of neurite outgrowth in ligand-stimulated cells expressing GFRα2b and the neuritogenic activities of the other two isoforms.

GFRα2b inhibited neurite outgrowth mediated by GFRα2a, GFRα2c, and GFRα1a isoform

Because GFRα2b transfectants did not induce neurite outgrowth when stimulated with ligands, we explored the possibility that this isoform may affect the morphological changes in cells coexpressing GFRα2b and GFRα2a or GFRα2c. We first established stably transfected Neuro2A cells coexpressing GFRα2a and GFRα2b (GFRα2a + GFRα2b) using a bicistronic vector. Expression and membrane targeting of GFRα2a were not affected when coexpressed with GFRα2b (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). As shown previously, ligand-induced stimulation of GFRα2a but not GFRα2b induced neurite outgrowth (Fig. 4*B*). Ligand-induced stimulation of cells coexpressing GFRα2a + GFRα2b showed significantly less neurite outgrowth (Fig. 5*A*). However, these cells extended neurite when treated with retinoic acid. Similarly, ligand stimulation of cells coexpressing GFRα2c and GFRα2b (GFRα2c + GFRα2b) showed significantly less neurite outgrowth (Fig. 5*A*).

Extending this finding, we next explored the possible inhibitory effect of the activation of GFRα2b on the neurite outgrowth induced by ligands in cells coexpressing GFRα1a. Cells expressing only GFRα1a showed significant neurite outgrowth when stimulated by GDNF, NTN, or retinoic acid (Fig. 5*B*). Interestingly, when stimulated by either GDNF or NTN, cells coexpressing GFRα1a and GFRα2b (GFRα1a + GFRα2b) showed significantly less neurite outgrowth. These observations indicate that the activation of GFRα2b inhibits neurite outgrowth induced by the activation of GFRα2a, GFRα2c, and even the structurally related GFRα1a.

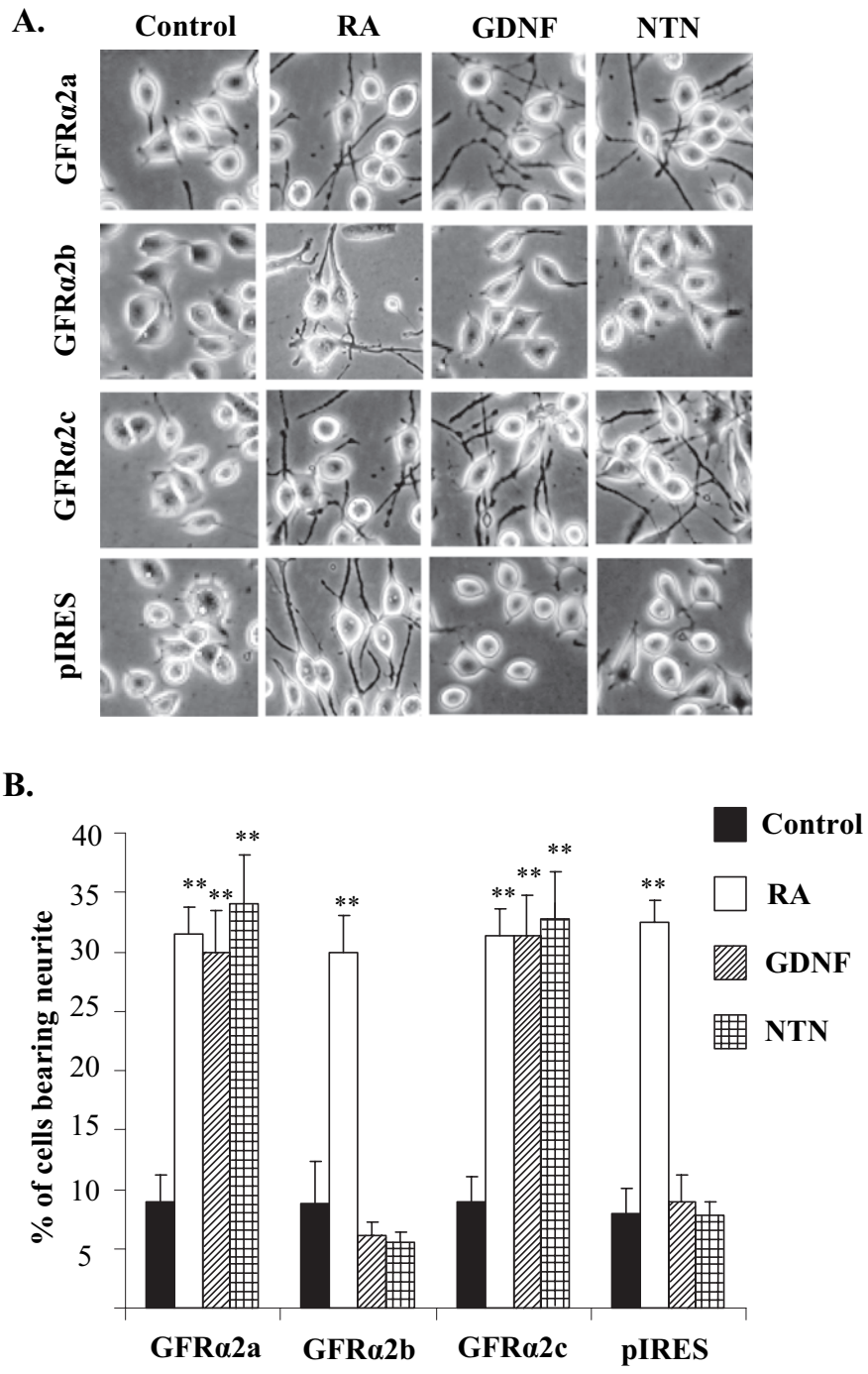

Figure 4. Differential neuritogenic activities of ligand-activated GFRα2 isoforms. Cells were seeded on six-well plates and incubated for 16–18 h in medium containing 10% serum. The cells were then exposed to GDNF or NTN (50 ng/ml) for 3 more days in 0.5% serum-containing medium. Retinoic acid (5 μM) was used as a positive control for cell differentiation. ***A***, Digital phase-contrast images of Neuro2A cells stably expressing GFRα2a, GFRα2b, GFRα2c, or pIRES vector control when treated with retinoic acid, GDNF, or NTN. ***B***, Percentages of cells bearing neurites that were at least twice the length of the cells bodies. More than 600 cells were counted per well, on at least three different fields. Experiments were repeated twice with three individual clones, with similar results. Significant differences in the percentage of cells bearing neurites between ligand stimulated and control were calculated using the paired Student's *t* test (*$p < 0.002$). Error bars indicate mean ± SD of triplicate measurements. RA, Retinoic acid.

Knock-down of GFRα2b resulted in an increase in neurite outgrowth

We next extended the above observation of the GFRα2b-induced inhibition of neurite outgrowth by investigating BE(2)-C cells

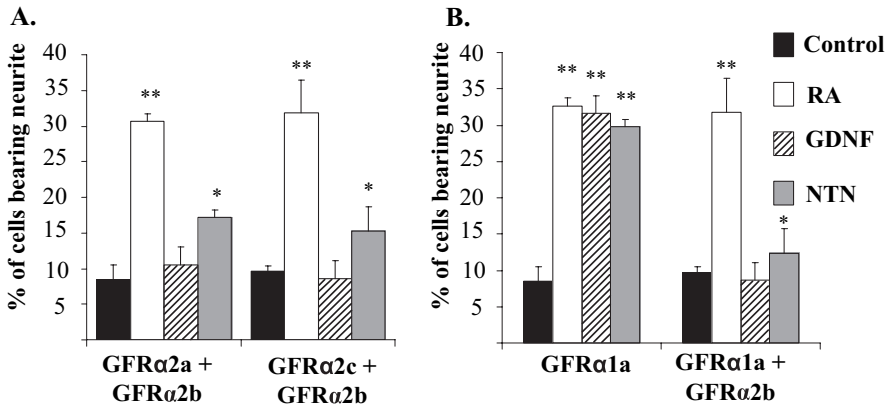

Figure 5. Ligand-induced neurite outgrowth in Neuro2A coexpressing GFRα2b with other GFRα isoforms. GFRα2b was stably coexpressed with GFRα2a (GFRα2a + GFRα2b) or GFRα2c (GFRα2c + GFRα2b) (***A***) or GFRα1a (GFRα1a + GFRα2b) (***B***). Cells were treated with or without GDNF or NTN (50 ng/ml) for 3 d in 0.5% serum-containing medium. Retinoic acid (RA; 5 μM) differentiated all the transfectants efficiently. Experiments have been repeated twice with three independent clones, with similar results. Significant differences in the percentage of cells bearing neurites between ligand stimulated and control were calculated using the paired Student's *t* test (**$p < 0.002$; *$p = 0.05$). Error bars indicate mean ± SD of triplicate measurements.

that have previously been shown to endogenously express GFRα2 receptors (Kobori et al., 2004). These cells express a comparable level of GFRα2a and GFRα2b (Fig. 6*A*). GFRα2c was found to be expressed at a level close to the detection limit of the assay (data not shown). The presence of GFRα2 but not GFRα1 in BE(2)-C cells agrees with previous observations (Kobori et al., 2004; Yoong et al., 2006) but not with a recent report using semiquantitative PCR (Hansford and Marshall, 2005).

Similar to the above observations with the coexpression of GFRα2b with GFRα2a, GFRα2c, or GFRα1a, both GDNF and NTN failed to induce neurite outgrowth in BE(2)-C cells (Fig. 6*C*, control). Neurite outgrowth was, however, observed when these cells were treated with retinoic acid, an indication that BE(2)-C cells have the capability of forming neurite-like structures. To test the hypothesis that the activation of GFRα2b may inhibit neurite outgrowth induced by GFRα2a or GFRα2c in BE(2)-C cells, we attempted to silence the expression of GFRα2b using siRNA. Because GFRα2b has no unique sequences compared with GFRα2a, the design of a GFRα2b isoform-specific siRNA poses a significant challenge. A series of siRNA duplexes were then designed with sequences overlapping exons 1 and 3 of GFRα2b (Fig. 6*B*). Of the five designs tested, only the siGFRα2b-13+7 showed significant discrimination in silencing GFRα2a and GFRα2b (Fig. 6*B*). This particular siRNA design, siGFRα2b-13+7, inhibited the expression of GFRα2b to <10% of the control, with no significant reduction in the expression of GFRα2a.

When GFRα2b expression was silenced, the BE(2)-C cells extended neurite-like structures when stimulated with either GDNF and NTN (Fig. 6*C*). This observation supports the notion that the activation of GFRα2b inhibits neurite outgrowth induced by ligand stimulation of GFRα2a.

Signaling and biochemical activities of GFRα2 isoforms in the co-expression model

To further investigate the signaling and biochemical events underlying ligand activation of GFRα2b in the coexpression model, we first examined the stimulation of MAPK (ERK1/2). GDNF stimulated ERK1/2 phosphorylation in GFRα2a or GFRα2c, but not GFRα2b, transfectants (Fig. 2*A*). In the coexpression models, both GDNF and NTN induced rapid and transient phosphorylation of ERK1/2 (Fig. 7*A*).

Interestingly, when stimulated with either GDNF or NTN, no change in the expression of either *egr-1* or *egr-2* was observed (Fig. 7*B–E*). However, significant upregulation of the expression of *fosB* was observed in the coexpression of GFRα2b with GFRα2a (GFRα2a + GFRα2b) or with GFRα2c (GFRα2b + GFRα2c). This observation showed that the activation of coexpressed GFRα2b with either GFRα2a or GFRα2c results in the activation of *fosB*, an early-response gene, reminiscent of that observed in GFRα2b transfected alone (Fig. 3).

GFRα2b also inhibited retinoic acid-induced neurite outgrowth and activated RhoA

We next addressed the possibility that GFRα2b may affect neurite outgrowth induced by retinoic acid, a non-GFL stimulus. Using Neuro2A-expressing GFRα2b, retinoic acid treatment resulted in extensive neurite outgrowth. Both GDNF and NTN dramatically reduced the number of cells bearing neurite-like structures in retinoic acid-treated GFRα2b transfectant (Fig. 8).

The Rho family of small GTPases and the associated regulators have been implicated in the modulation of neurite formation, axonal pathfinding, and dendritic arborization (Mackay et al., 1997; Van Aelst and Cline, 2004). Thus, it was of interest to examine the possibility that GFRα2b may activate the Rho family of GTPases. When stimulated with either GDNF or NTN, Neuro2a coexpressing GFRα2a and GFRα2b (GFRα2a + GFRα2b) or GFRα2c and GFRα2b (GFRα2c + GFRα2b) did not extend neurite-like structures (Fig. 5*A*). However, a significant number of these cells extended neurite-like structures in the presence of C3 transferase, suggesting the involvement of the Rho family of GTPases in the inhibitory effects of GFRα2b (Fig. 9*A*,*B*). Because Neuro2A cells have previously been shown to respond to LPA, resulting in the inhibition of neurite outgrowths through the RhoA-dependent mechanism (Sayas et al., 2002), it was not surprising that C3 transferase was found to inhibit LPA effects on retinoic acid-induced neurite outgrowth. At the concentration of C3 transferase used in this study, no significant cell death was observed (data not shown).

To gain a better understanding of the mechanisms underlying the inhibitory effects of GFRα2b, we next examined the possible involvement of ROCK, which is known to be an effector of RhoA in the negative regulation of neurite outgrowth (Dickson, 2001; Sayas et al., 2002). Using the ROCK inhibitor Y27632, the inhibitory activity of LPA on retinoic acid-induced neurite outgrowth was significantly attenuated (Fig. 9*A*,*B*). However, the same concentration of Y27632 (10 μM) did not attenuate the inhibitory activity of GFRα2b (Fig. 9*A*,*B*). Higher concentrations of Y27632 (20 μM) resulted in significantly higher background neurite outgrowth and therefore complicated the interpretation of the study.

To investigate the possible involvement of RhoA in the inhibitory effects of GFRα2b, an attempt was made to pull down activated RhoA from cells lysates using glutathione *S*-transferase (GST)–Rhotekin and subsequently immunoblotted for RhoA. Similar to the effects of LPA, GFRα2b, when stimulated with either NTN or GDNF, was found to activate RhoA significantly (Fig. 9*C*). However, Neuro2A expressing GFRα2a, GFRα2c, or

pIRES vector control did not activate RhoA significantly when stimulated with these ligands. This observation is consistent with the suggestion that RhoA and/or other Rho GTPases may be involved in the inhibition of neurite outgrowth mediated through GFRα2b.

The involvement of Rho in the activation of GFRα2b is not restricted to inhibiting GFRα1a-, GFRα2a-, or GFRα2c-induced neurite outgrowth but also to that induced by retinoic acid (Fig. 10*A*). Similar to the above observations, the inhibitory effects of GFRα2b on retinoic acid-induced neurite outgrowth appeared to be mediated through a Rho-dependent manner. Furthermore, the inhibition of ROCK may be sufficient to oppose the effects of LPA but not that of GFRα2b on retinoic acid-induced neurite outgrowth (Fig. 10*B*).

Discussion

This study demonstrates a novel function of GFRα2b, an alternatively spliced isoform of GFRα2. When activated by ligands (GDNF or NTN), GFRα2b inhibited neurite outgrowth induced by GFRα1a, GFRα2a, and GFRα2c isoforms. Furthermore, GFRα2b was found to inhibit a non-GFRα stimulus, retinoic acid-induced neurite outgrowth, and to activate RhoA.

Alternative splicing is prevalent in many mammalian genomes and is a means of producing functionally diverse polypeptides from a single gene (Blencowe, 2006). Recently, genome-wide microarray and large-scale computational analyses of expressed-sequence tag and cDNA sequences have estimated that >50% of human multi-exon genes are alternatively spliced (Modrek and Lee, 2002). Comparative genomic analyses also demonstrated that the greatest amount of conserved alternative splicing occurs in the CNS (Kan et al., 2005). In many systems, alternative splicing events have been shown to produce isoforms with distinct activities and biochemical properties, as a means for diverse biological functions (Lee and Irizarry, 2003).

In the cortex of human, mouse, and rat brain, the expression of GFRα2 mRNA has been reported (Sanicola et al., 1997; Widenfalk et al., 1997; Golden et al., 1998, 1999; Trupp et al., 1998). However, the probes used in these studies cannot distinguish the expressions of the isoforms. In the present study, we were able to specifically amplify all three isoforms in the human brain regions using exon-overlapping primers (Too, 2003). In the human brain, all three GFRα2 isoforms are expressed at comparable levels, with GFRα2c significantly higher than the other two isoforms. Compared with the other regions of the human brain, the cortex expressed the highest levels of the isoforms. The functional significance of these isoforms in the cortex has yet to be defined. Interestingly, the high expressions of the GFRα2 isoforms in the cortex, a region of the brain involved in learning complex tasks, and the observation that GFRα2 knock-out mice show significant impairment in several memory tasks (Voikar et al., 2004) may suggest a possible role of GFRα2 signaling in the development and/or maintenance of cognitive abilities that help in solving complex learning tasks.

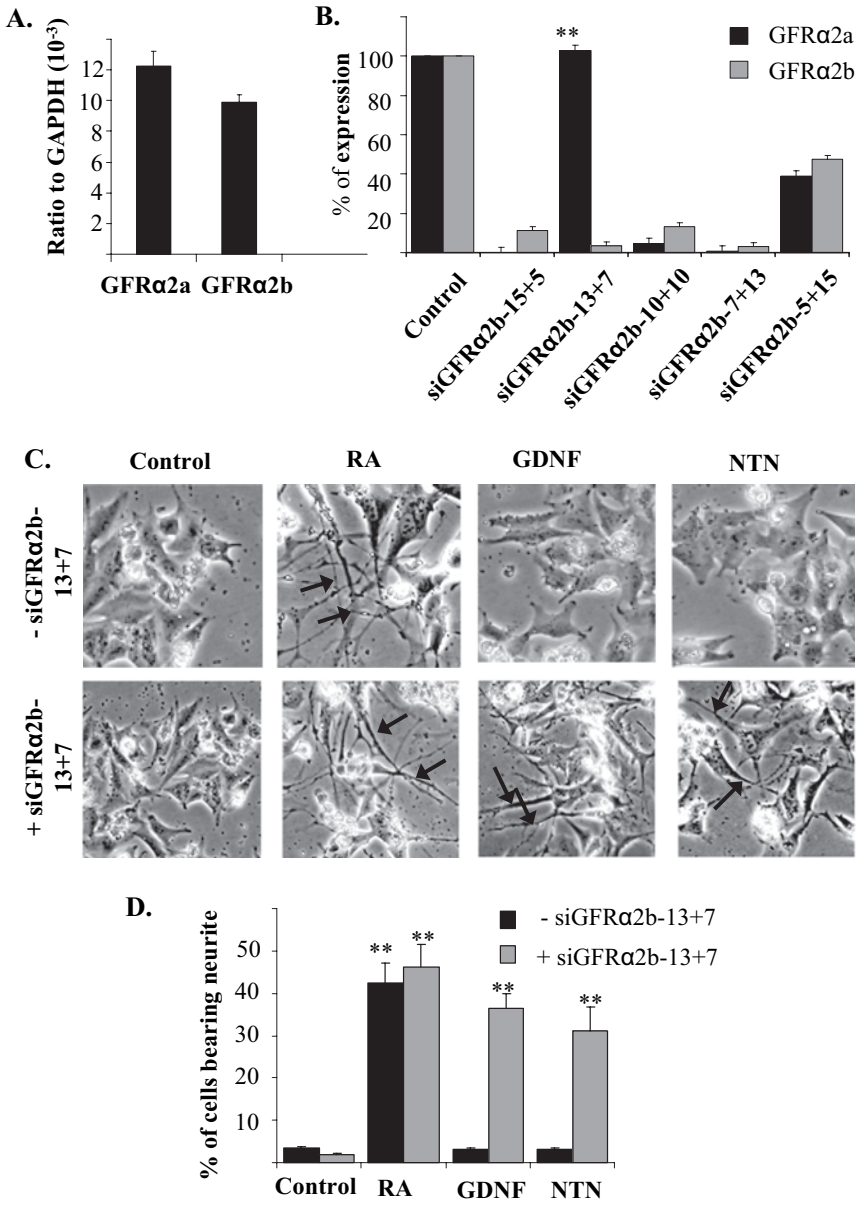

Figure 6. Silencing of GFRα2b expression in human BE(2)-C cells. ***A***, The expression levels of GFRα2a and GFRα2b in BE(2)-C cells were determined using quantitative real-time PCR. ***B***, Effects of various designs of siRNA sequences on the expressions of GFRα2a and GFRα2b in BE(2)-C. siRNA duplex (20 pmol) was transfected into cells, and total RNA was harvested 6 h later. The expressions of GFRα2a and GFRα2b were then measured by quantitative real-time PCR. Significant differences between the expression of the two isoforms after silencing with each of the siRNA designs were calculated using the paired Student's *t* test (**$p = 0.001$). ***C***, ***D***, Neurite outgrowth of BE(2)-C cells after silencing of GFRα2b. ***C***, Top row, Cells were stimulated with retinoic acid (5 μM), GDNF, or NTN (50 ng/ml) in the absence of siRNA. Bottom row, Cells were transfected with siGFRα2b-13+7 for 6 h and subsequently stimulated with retinoic acid (5 μM), GDNF, or NTN (50 ng/ml). Pretreament of cells with siGFRα2b-13+7 and subsequent stimulation with GDNF or NTN resulted in the formation of neurite-like structures (arrows). ***D***, Percentages of cells bearing neurites that were at least twice the length of the cells bodies were scored in the presence (+) or absence (−) of the siRNA siGFRα2b-13+7. Similar results were obtained from replicates of three individual experiments. Significant differences in the percentage of cells bearing neurites between ligand stimulated and control were calculated using the paired Student's *t* test (**$p < 0.002$; *$p = 0.05$). Error bars indicate mean ± SD of triplicate measurements. RA, Retinoic acid.

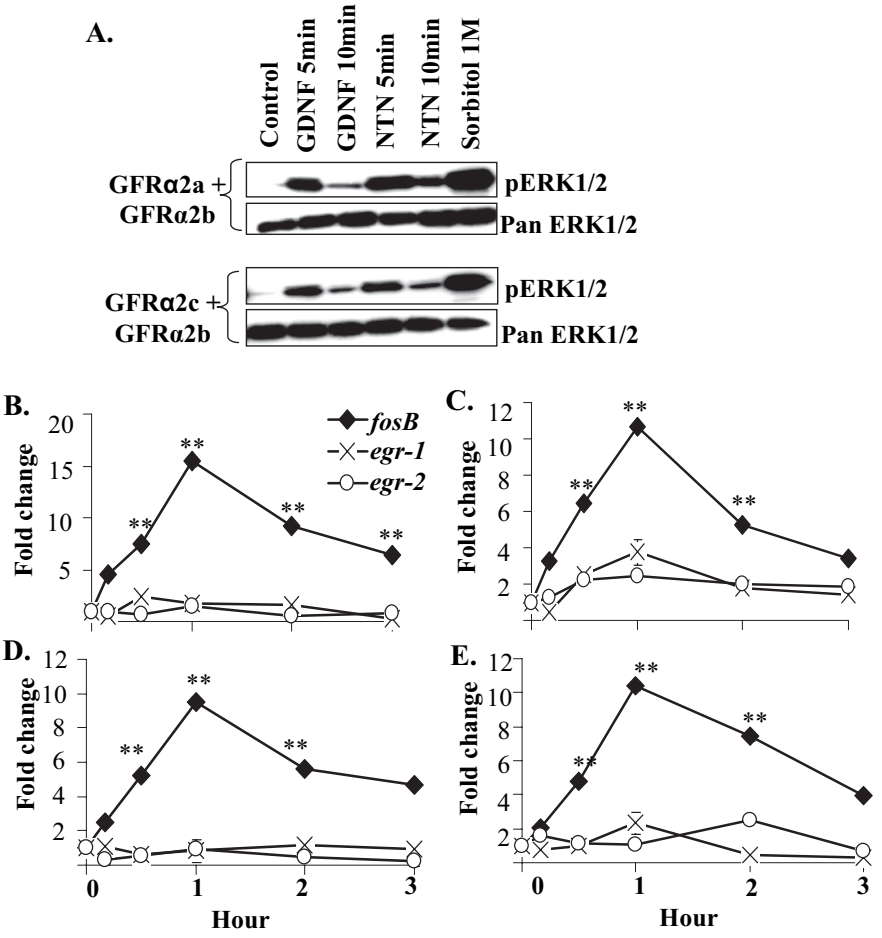

Figure 7. Ligand-regulated ERK1/2 signaling and expressions of immediate-early-response genes in Neuro2A coexpressing GFRα2b and the other GFRα2 isoforms. ***A***, Western blot analyses of the activation of ERK1/2. Neuro2A cells stably coexpressing the isoforms GFRα2a and GFRα2b (GFRα2a + GFRα2b) or GFRα2c and GFRα2b (GFRα2c + GFRα2b) were treated with GDNF, NTN, or Sorbitol for the period of time indicated. Phospho-specific antibodies to ERK1/2 were used for detection, and the blots were reprobed with pan antibody serving as controls for protein loadings. ***B–E***, Kinetic analyses of GDNF- or NTN-regulated expressions of early-response genes in the coexpression models. Expressions of *fosB*, *egr-1*, and *egr-2* were measured with quantitative real-time PCR in cells stably coexpressing GFRα2a with GFRα2b (GFRα2a + GFRα2b) when stimulated with GDNF (***B***) or NTN (***C***); cells stably coexpressing GFRα2c with GFRα2b (GFRα2c + GFRα2b) were stimulated with GDNF (***D***) or NTN (***E***). Significant differences in expression of genes between ligand stimulated and control were calculated using the paired Student's *t* test. A value of $p < 0.05$ was considered significant (**$p < 0.001$).

GDNF and NTN are known to similarly activate a number of signaling pathways, including ERK, phosphatidylinositol 3-kinase/AKT, p38 MAPK, and JNK (Trupp et al., 1999; Takahashi, 2001; Pezeshki et al., 2003; Ichihara et al., 2004), and regulate the expressions of various immediate-early-response genes (Fukuda et al., 2003; Pezeshki et al., 2003). In this study, it is intriguing to note that the activation of specific signaling pathways but not the early-response genes is dependent on the ligands used. For instance, GDNF was found to potently activate ERK1/2 through GFRα2a and GFRα2c in a dose- and time-dependent manner but did not activate GFRα2b significantly. This was not attributable to the failure of GDNF to interact with GFRα2b because GDNF displaced bound [^{125}I]GDNF equally well with all three isoform transfectants. Similarly, GDNF activated AKT through GFRα2b and GFRα2c but not through GFRα2a. However, NTN showed similar activations of ERK1/2 and AKT through all of the three isoforms. GDNF at lower concentrations appeared to be slightly more potent than NTN in the activation of ERK1/2 but not at higher concentrations in GFRα2a and GFRα2c transfectants. The significance of this, however, is unclear presently.

Both GDNF and NTN have previously been shown to have similar properties in activating the multicomponent receptor complex (Baloh et al., 1997; Airaksinen et al., 1999; Wang et al., 2000; Scott and Ibanez, 2001; Coulpier et al., 2002; Charlet-Berguerand et al., 2004). In addition, midbrain dopaminergic neurons that only express GFRα1 appear to survive equally well with both GDNF and NTN *in vitro* and *in vivo* (Horger et al., 1998). However, there are observations of distinct functional differences with the use of specific ligands. Although GDNF and NTN promote the survival of dopaminergic neurons through GFRα1 (Cacalano et al., 1998; Akerud et al., 1999), only GDNF possess neuritogenic and hypertrophic effects (Akerud et al., 1999). In cultured sympathetic neurons, GDNF was able to promote the survival of culture sympathetic neurons through GFRα2, but NTN could not promote survival through GFRα1 (Buj-Bello et al., 1997). Furthermore, GDNF but not NTN could promote the axonal growth of DRG neurons through GFRα1 (Paveliev et al., 2004). Consistent with these studies, recent observations show differential ligand signaling through the activation of GFRα1 (Lee et al., 2006) and distinct activation of microRNAs by specific ligands through the GFRα2 receptor complexes (Yoong et al., 2006), supporting the emerging view that cross talk of exogenously applied GDNF and NTN with a specific receptor may, in some instances, result in distinct functions.

It is well documented that GDNF and NTN are potent trophic factors that have potent effects on neuronal differentiation and promote survival and sprouting of ventral mesencephalic dopaminergic neurons in primary cultures and other neuronal cultures (Lin et al., 1993; Akerud et al., 1999; Baloh et al., 2000; Yan et al., 2003; Wanigasekara and Keast, 2005; Zihlmann et al., 2005). The finding in this study of a particular alternatively spliced variant of GFRα2 inhibiting neurite outgrowth was unexpected. Unlike GFRα2a and GFRα2c, GFRα2b transfectants did not induce neurite outgrowth when activated by either GDNF or NTN. Both GFRα2a and GFRα2c (but not GFRα2b) activated the early-response gene *egr1* (also known as NGFI-A, krox-24, zif-268, and TIS-8), consistent with a role of *egr1* in neuronal differentiation (Pignatelli et al., 1999; Knapska and Kaczmarek, 2004). In coexpression studies, GFRα2b was found to inhibit ligand-induced neurite outgrowth by GFRα2a and GFRα2c. Similarly, in BE(2)-C cells endogenously expressing GFRα2b isoform, both GDNF and NTN did not significantly alter the morphology of the cells. However, the silencing of GFRα2b and subsequent treatment with either GDNF or NTN caused the cells to extend neurite-like structures.

Interestingly, in coexpression studies, *fosB* was upregulated and paralleled the upregulation of the immediate-response gene observed in GFRα2b transfectants by GDNF or NTN. It is not known whether the coexpression of GFRα2b with GFRα2a or GFRα2c may have affected the protein expression levels of the latter two spliced variants resulting in the attenuation of neurite extension on ligand stimulation. The possibility of GFRα2b affecting the expression of the other spliced variants and the effects of expressions levels is currently investigated.

The inhibition of neurite outgrowth by GFRα2b is not restricted to the GFRα2 family of isoforms. Ligand-activated GFRα2b also inhibited the neurite outgrowth induced by GFRα1a, another member of GFR. Intriguingly, the activation of GFRα2b inhibited neurite outgrowth induced by retinoic acid. The underlying GFRα2b inhibitory mechanism appears to involve the Rho family of GTPases. RhoA is a member of the Rho GTPase family, which includes RhoA, Rac, and Cdc42 (Luo, 2000; Van Aelst and Cline, 2004). Although Rac and Cdc42 have been shown to be involved in promoting neurite and axonal outgrowth, RhoA has been the focus in studies of molecular mechanisms for some glia-derived neurite outgrowth inhibitory factors such as Nogo-A, myelin-associated glycoprotein (Niederost et al., 2002), and LPA (Sayas et al., 2002). More recent findings have revealed that RhoA mediates neurite outgrowth inhibition by reorganization of actin and the microtubular network (Dickson, 2001; Leung et al., 2002). Consistent with these findings is that GDNF and NTN increased the active form of RhoA in GFRα2b but not GFRα2a or GFRα2c transfectants. Furthermore, the *Clostridium botulinum* C3 exoenzyme specifically ADP-ribosylates and inactivates Rho, thereby increasing neurite outgrowths in GFRα2a/GFRα2b and GFRα2c/GFRα2b coexpression models. It is interesting to note that GDNF induced RET-mediated phosphorylation of focal adhesion kinase, paxillin, and p130C through the activation of the Rho family of GTPase and inhibited the outgrowth of neurites in TGW-I-nu cells (Murakami et al., 1999). It is, however, unclear whether this observation is mediated through GFRα2b.

Compared with GFRα2a, both GFRα2b and GFRα2c showed deletion of eight cysteine residues and *N*-glycosylation sites at the N terminus (Wong and Too, 1998). GFRα2 is thought to be structurally organized into three distinct domains. The N-terminal domain has previously been shown to be dispensable for ligand binding specificity and RET phosphorylation of GFRα receptors (Scott and Ibanez, 2001). Extending this observation, the N-terminal domain encoding the unique sequences of GFRα2a, GFRα2b, and GFRα2c may serve to regulate distinct biochemical and cellular processes. It is tempting to speculate that the expression and interactions of specific GFRα2 receptor spliced isoforms may play an important role in neuronal differentiation involving GDNF and NTN. The recent observation in which the expressions of GFRα2 isoforms are differentially regulated in Nurr1-induced dorpaminergic differentiation of embryonic stem cells is consistent with this suggestion (Sonntag et al., 2004).

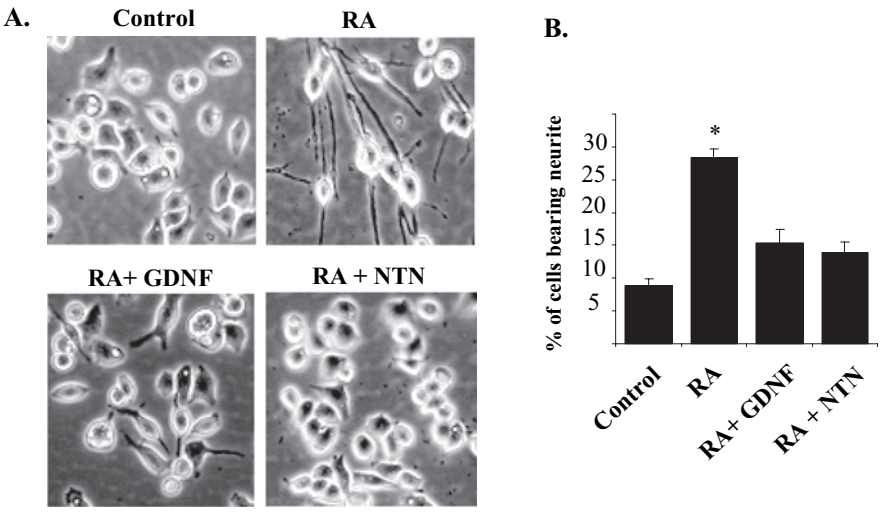

Figure 8. Ligand-activated GFRα2b antagonizes the neurite outgrowth induced by retinoic acid (RA). RA (5 μM) induced neurite outgrowth in GFRα2b-expressing Neuro2A cells. When treated together with GDNF or NTN (50 ng/ml), neurite outgrowth induced by RA was significantly attenuated. ***A***, Phase-contrast images of Neuro2A cells stably expressing GFRα2b, treated with RA, GDNF, or NTN for 3 d. ***B***, Graph of the percentage of cells bearing neurite with at least two times the length of the cell bodies and the effects of RA, GDNF, and NTN. Similar results were obtained from three independent clones. Significant differences in the percentage of cells bearing neurites between ligand stimulated and control were calculated using the paired Student's *t* test (*$p <$ 0.002). Error bars indicate mean ± SD of triplicate measurements.

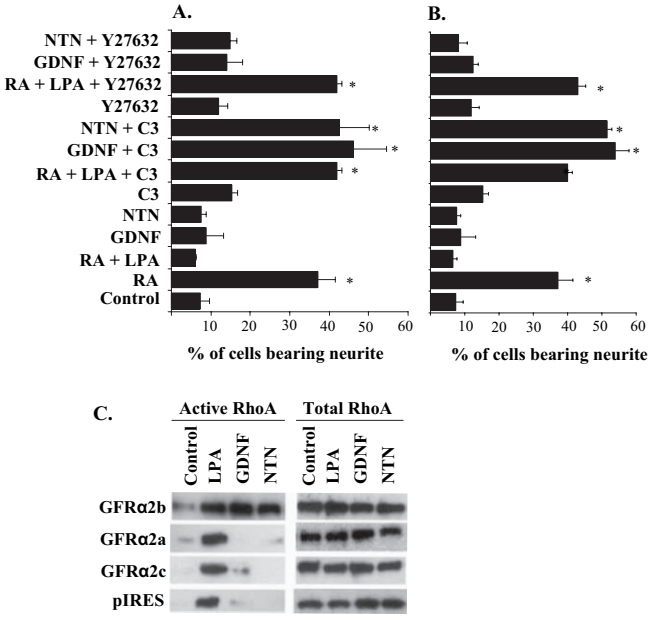

Figure 9. Effects of RhoA and ROCK inhibitors in ligand-induced neurite outgrowth of GFRα2 isoform coexpression models and the ligand-induced activation of RhoA in GFRα2 isoform transfectants. ***A***, ***B***, Effects of RhoA inhibitor exoenzyme C3 transferase (1 μg/ml) and ROCK inhibitor Y27632 (10 μM) on ligand-induced neurite outgrowth in coexpression models of GFRα2a and GFRα2b (***A***) or GFRα2c and GFRα2b (***B***). LPA was used as a positive control in this study. LPA (10 μM) antagonizes neurite outgrowth induced by 5 μM retinoic acid (RA); such neurite inhibition of LPA was attenuated by C3 (1 μg/ml) and Y27632 (10 μM). The means ± SD were calculated from results obtained in triplicates. The effects of RhoA and ROCK inhibitors were compared with the effects of the inhibitors alone. With the concentrations of inhibitors used, no significant cell deaths were observed. Significant differences in the percentage of cells bearing neurites were calculated between ligand stimulated and control, using the paired Student's *t* test (*$p \leq 0.01$). ***C***, Analyses of RhoA activation in Neuro2A cells transfected with GFRα2 isoforms or pIRES control. After a 10 min pretreatment of LPA (10 μM), GDNF, or NTN (50 ng/ml), GTP-bound RhoA was pulled down from cell lysates using GST–Rhotekin and immunoblotted for RhoA. LPA served as a positive control for RhoA activation. Blotting of total RhoA in cell lysates showed similar loading of cell lysates.

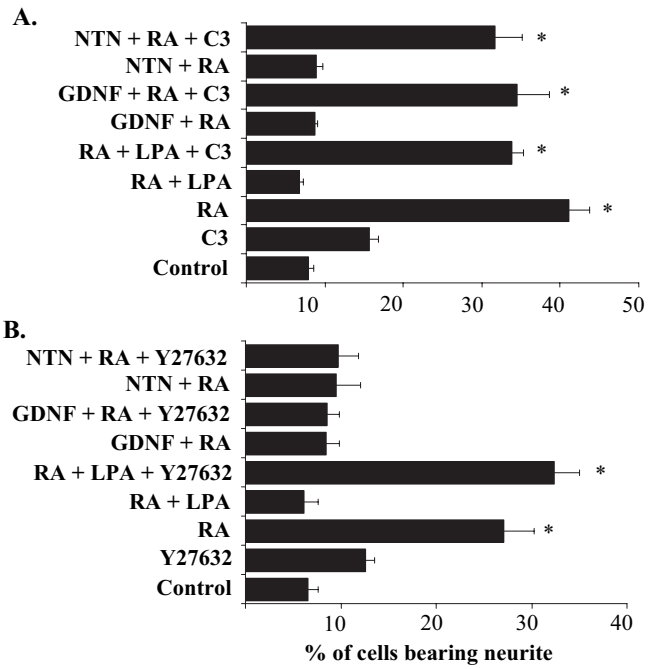

Figure 10. Effects of RhoA and ROCK inhibitors on GFRα2b inhibition of retinoic acid (RA)-induced neurite outgrowth. ***A***, RhoA inhibitor exoenzyme C3 transferase (1 μg/ml) inhibited the ligand-activated GFRα2b attenuation of neurite extension induced by RA (5 μM). The same concentration of exoenzyme C3 transferase also attenuated LPA (10 μM) inhibition of RA-induced neurite extension. ***B***, Lack of effect of ROCK inhibitor Y27632 on the ligand-activated GFRα2b inhibition of RA induced neurite extension. The same concentration of Y27632 (10 μM) significantly attenuated the neurite outgrowth inhibition induced by LPA. The means ± SD were calculated from results obtained in triplicates. The effects of RhoA and ROCK inhibitors were compared with the effects of the inhibitors alone. With the concentrations of inhibitors used, no significant cell deaths were observed. Significant differences in the percentage of cells bearing neurites were calculated between ligand stimulated and control, using the paired Student's *t* test (*$p \leq 0.01$).

In summary, this study provides the first evidence that GDNF and NTN have distinct neuritogenic effects mediated through specific GFRα2 isoforms. GFRα2b inhibited GFRα1 and GFRα2, and retinoic acid mediated neuritogenesis through the Rho family of GTPases. The emerging view is that the combinatorial interactions of the spliced isoforms of GFRα2, RET, and NCAM may contribute to the complexity of a multicomponent signaling system and may produce the myriad of observed biological responses.

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